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I.

GLAND-CELLS OF INTERNAL SECRETION IN THE SPINAL CORD OF THE SKATES.

By CARL CASKEY SPEIDEL,
Of Princeton University.

Nine plates; three text figures.

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GLAND-CELLS OF INTERNAL SECRETION IN THE SPINAL CORD OF THE SKATES.

By Carl Caskey Speidel.

INTRODUCTION.

Ever since the discovery of the electrical apparatus of the skate by Stark in 1844, it has been the subject of investigation by many workers. Among these may be mentioned particularly Leydig, Remak, Ecker, Köllicker, Schultze, Babuchin, Sanderson and Gotch, Ewart, and Ballowitz. Most of these investigators dealt chiefly with the histology of the electric organs and the manner in which the nerves terminate upon the electric disks. Babuchin discovered that the electric organs were derived from embryonic muscle-tissue.

Sanderson and Gotch made an experimental study of the physiology of the electric organs. They exposed and stimulated various parts of the brain and also of the body-surface of the skate, and drew the conclusion that an electrical center existed in the optic lobes. No morphological work was done.

The first one to describe the position of the electric motor cells was J. C. Ewart, who had previously described the histogenesis of the electric tissue. Reasoning that the electric motor cells should be placed near to the electroplaxes which they innervate, he correctly located these structures in the lower part of the spinal cord. He figured these cells as resembling in form the ordinary type of muscle motor nerve-cells. The chief difference was in size, the electric motor cells being much larger than the muscle motor cells. He found that these electric motor cells were present in the spinal cord only in the region opposite to the electric organs. He made no mention, however, of certain large, peculiar cells which appear in this region.

A. Romano studied the motor electric centers of the selachian fishes, but evidently did not see or accurately describe these cells of the skates. They were first noticed and described by U. Dahlgren, and the question at once arose as to their function. Dahlgren suggested that these cells might be the real electric motor cells, and it was with this as a working hypothesis that this work was begun.

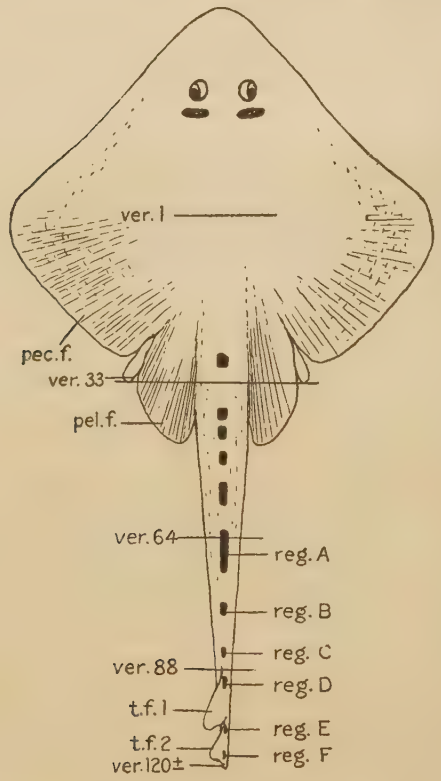
The skates used in this study were collected for the most part along the coasts of New Jersey, Massachusetts, and Maine. The experimental work was done chiefly with the species *Raia ocellata*, the common spotted skate. However, *Raia lævis*, *Raia erinacea*, and *Raia radiata* were also studied. Besides these, valuable embryological material was turned over to the writer by Professor Dahlgren, who had

collected and prepared skate embryos of the species *Raia punctata* at the Zoological Station at Naples.

I wish to express my appreciation and thanks for the facilities for research offered me by the Harpswell Laboratory, Maine, during the summer of 1915; by the United States Bureau of Fisheries, Woods Hole, Massachusetts, during the summer of 1916; and by the Marine Biological Laboratory, Woods Hole, Massachusetts, during the summer of 1916 and May 1917. I am greatly indebted to Professor E. G. Conklin for criticism and advice throughout this work. Most of all do I wish to acknowledge my thanks to Professor Ulric Dahlgren, whose help and guidance have made this work possible.

DISTRIBUTION.

A study of the spinal cord of the skate was first made in order to determine the exact anterior-posterior distribution of these large cells of unknown function. It had been noticed by Dahlgren that the cells



TEXT-FIGURE 1.—Diagram of the skate to show the region of the tail in which the large cells of the spinal cord are found. The anterior limit of the cells is indicated by the line drawn through the tail at the level of the sixty-fourth vertebra. The anterior limit of the electric organs is indicated by the line drawn through the root of the tail at the level of the thirty-third vertebra. The shaded regions represent the parts of the spinal cord examined in the skate in which the calculation of the total number of these large cells was made.

pec. f. pectoral fin. *ver. 1.* first vertebra.
pel. f. pelvic fin. *ver. 33.* thirty-third vertebra.
reg. A. region A. *ver. 88.* eighty-eighth vertebra.
t. f. 1. first tail-fin *ver. 120* ± one hundred and twentieth vertebra, approximately.
t. f. 2. second tail-fin.

TABLE 1.

Region.	At level of—	No. of cells per mm.
A.....	64 to 70 vertebræ.	0.324
B.....	74 to 78	2.302
C.....	84 to 87	2.250
D.....	90 to 92	4.755
E.....	105 to 107	3.470
F.....	112 to 115	3.380

were always found in regions of the spinal cord that were opposite to the electric organs, none appearing in that part anterior to the electric organs.

The electric organs of *Raia ocellata* extend throughout the tail, one on each side of the vertebral column. The anterior limit is about at the level of the thirty-third vertebra, or just anterior to the insertion of the pelvic fins. The posterior limit is the extreme tip of the tail. In the adult skate there are about 120 vertebræ. Sections of the spinal cord were taken at various levels, as indicated by the shaded regions in text-figure 1. As a result, it was found that the cells in question were present in the spinal cord only from the level of the sixty-fourth vertebra to the tip of the tail.[1] They do not, therefore, correspond in anterior-posterior distribution with the extent of the electric organs. They are situated opposite the caudal portion only of the electric organs. Any explanation of the function of these cells must take into account their peculiar distribution in the posterior part of the spinal cord only.

The cells were found to vary in frequency in different parts of the spinal cord. Counts were made of the number of cells in each of the regions indicated in the figure, and a calculation of the number of cells per millimeter was made in each case. These results are summed up in table 1. The cells are seen to be most numerous near the tip of the tail in the region of the two little tail-fins. No arrangement of the cells corresponding to the segmental structure of the tail was apparent. A calculation of the total number of these cells showed that there were about 600. It is interesting to compare this with the number of electroplaxes in the electric organs of a large skate—20,000, as estimated by Ewart.

MORPHOLOGY.

A typical cross-section of the spinal cord of *Raia ocellata* in the region of these large cells is shown in plate 1, figure 1. The spinal cord is enveloped by the meninx primitiva, which carries the blood-vessels. The spinal vein is present on the dorsal side and the spinal artery on the ventral side. The central canal is situated more or less ventrally. The cells are found on each side of the central canal, lying on the anterior edge of the gray matter. In this same section may be seen a nerve-cell, probably an electric motor nerve-cell.

One of the most striking things about these remarkable cells is their great size. In bulk they average about 20 times the volume of the muscle nerve-cells. If they were nerve-cells they would be among the largest nerve-cells known. They are very irregular in shape, but may be described as somewhat elongated and slightly flattened cells averaging in *Raia punctata* about 300 mm. long, 200 mm. wide, and 176 mm. thick. The greater length lies in an anterior-posterior position in the cord, the width is lateral, and the thickness is dorso-ventral. These measurements do not take into account large local irregularities in the

[1]The anterior limit varies somewhat in the different species of skates. In *Raia lævis* and *Raia punctata* it is a few vertebræ farther forward.

shape of the cells. Each cell usually has a few large, heavy processes which extend mainly in two directions. One of these is downward and slightly medial, while the other is lateral, the process or processes marking a distinct boundary in transverse sections of the cord between an anterior and posterior portion of the white matter or fiber bundles of the region. In addition to these larger lateral processes, there were other and smaller processes which passed out anteriorly and posteriorly from the ends of the somewhat elongate cell. These smaller processes were not always observed. The position of these cells in cross-sections of the spinal cord is quite variable. Figure 1 shows two of them very close to the central canal. They may, however, be removed from it by some distance, as is the case in figure 21 (plate 5).

The nucleus is of extraordinary structure, and aside from the large size of the cell is the most prominent feature to attract the attention when the cell is first examined (fig. 5, plate 2). At first sight it appeared to be multiple in character and of marked irregularity, but when the series of sections of any particular cell was carefully examined, and when cells were reconstructed by wax plates or by graphic methods, it was seen that it was in reality but a single nucleus of the distributed type. Figure 3, plate 2, is a photograph of a wax reconstruction of the nucleus of a single cell, the same cell shown in cross-section in figure 5.

There sometimes appears to be a center from which the principal masses of the nucleus branch, and this center is probably the original center. It is best seen in cells of *Raia punctata*. It is conspicuous for its lack of chromatic matter. This center is usually round in its general outline, like the nucleus of a muscle motor nerve-cell, and from it the various branches of the nucleus are given off, beginning as narrow tube-like arms and branching in an irregular way as they pass toward the periphery. They get wider as they reach out from the center and at their terminal parts they enlarge into rounded portions, each of an equal or greater volume than the central part of origin. The extent of distribution varies, but its branches extend well towards the limits of the cell. Often the whole nucleus, expecially the central part, lies at one side and the branches embrace a central core of cytoplasm. Some nuclei have fewer and larger branches than others.

The nucleus is separated from the cytoplasm by a very well-defined nuclear membrane, which is always continuous and intact. It has some thickness and staining capacity and its refractive index is higher than of most of the other cell-substances.

The achromatic substance of the nucleus, the so-called nuclear sap, is about as thin or fluid in composition as in the usual nerve-cell. When fixed it is thrown into a reticular precipitate varying according to the fixative used, which serves to hold the numerous chromatic granules of the nucleus in their approximately true positions. This achromatic substance acts very much as mucin, not staining with iron

hematoxylin, but taking mucicarmine and Delafield's hematoxylin very deeply. The corresponding achromatic nuclear content of the muscle motor cells does not act so with regard to mucin stains and on the same slide is clear or takes an acid counterstain like eosin. With a Delafield hematoxylin stain the chromatin contents of the nucleus are masked or entirely hidden by the deeply staining nuclear sap. With a bulk stain like borax carmine it also stains deeply, while in the muscle motor cells it shows no staining power. With the Pal-Weigert staining method the chromatic substance of the nucleus becomes a deep brown. The chromatin of the muscle motor nerve-cells, however, remains unstained. It is clear from these reactions that the nucleus contains a chromatic constituent not found in the nerve-cells of this animal.

The size or volume of this nucleus is large even in proportion to the immense bulk of the cell. The central area alone approximates in size the large nuclei of nerve-cells in the same sections. Each of the numerous terminal portions of the various branches alone is larger than the central portion, and if the whole nucleus were to be concentrated into the usual spherical form it would certainly be among the largest nuclei known.

When well-fixed Flemming material is stained with a sharp regressive stain like iron hematoxylin, the real chromatic material of the nucleus comes out strongly, and we find that the largest quantity of such material consists of very numerous, evenly distributed granules of considerable size. This size is fairly even and uniform for the larger particles, and scattered among them are very small particles together with a few intermediate in size. All parts of the nucleus possess these granules, except sometimes the central area of the nucleus, which may be almost clear. In many cases one or two much larger chromatic bodies are found in the central area or in that part of one of the branches nearest to it. These chromatin particles are not round bodies, although they are compact. They are elongate to varying degrees, but can not be called rod-like. While they are also irregular in shape, their outline is smooth and not jagged or rough, except in a few cases.

The cytoplasm of these cells was quite homogeneous in character when found at rest. That it had a chromophilic content was quite apparent, for it stained with the proper stains and to sufficient depth to show this. But in no specimen was this shown to be organized into Nissl granules. In the neighboring motor nerve-cells the chromophilic substance is plainly visible as Nissl granules which are assembled at or near the periphery of the cell in the form of typical tigroid bodies. Furthermore, the general color of the cytoplasm outside these tigroid bodies is far lighter than the cytoplasm of the large cells in question.

No definite neurofibrils are present. It was thought at first that with the proper stains some of the many processes of these cells might

be demonstrated to contain neurofibrils. Accordingly, several special methods of neurological technic were employed in preparing sections, including the Cajal, Paton-Bielschowsky, Golgi, Shunda, and Pal-Weigert methods. In no case were definite neurofibrils detected.

Another structure or series of structures seen in the cyptoplasm of these cells was at first taken to be pathological in character, but when seen in the cells of all the skates examined (three Italian species at Naples,[1] several English species, and four American species) it was concluded that no phenomena so constant in their presence could be abnormal. The structures referred to are vacuoles, granules, and precipitates of a constant and specific structure found in the cytoplasm of the cells and in the surrounding tissues. The cell in figure 5 shows a few vacuoles in the cytoplasm and a large number of granules, particularly along the processes. Not all of the cells were found to show the vacuoles, and some showed many more than others, even in the same individual. A few typical vacuolated cells are shown in plate 3. Figures 6 and 7 are taken from a skate of the species *Raia punctata*, and figure 8 from a skate of the species *Raia lævis*.

The history of the vacuoles in the cells of *Raia punctata* appears to be as follows: In their earliest stages they are small and very numerous and are found principally in the larger masses of cytoplasm of the cell. Due to the distributed and often peripheral arrangement of the nuclear material, these larger masses of cytoplasm are usually in the central part of the cell. The vacuoles grow in size and become less numerous, indicating that a coalescence takes place to form fewer larger structures. Figure 6 shows such a condition with one vacuole of considerable size and groups of much smaller ones. When thus increased in size they show at first an empty cavity which indicates the presence of some solution or soluble material in life. The cavity is lined with a fine membrane of delicate nature separating the cytoplasm from the contents of the vacuole.

In a more advanced stage the vacuoles are all large, in some cases excessively large, and they are usually near the periphery of the cell-body. At this time it is often possible to see a slight acid-staining precipitate in them having a definite fixation reticulum[2] (fig. 7). In what is taken to be a slightly later stage, figure 8, small round granules have made their appearance in the precipitate, and these granules grow in numbers and also in size. The number and size of the granules in ripe vacuoles varies greatly. Some vacuoles may have many small granules. In one average-sized vacuole of a cell of *Raia lævis* containing small granules, the number of granules was found to be 180 (the estimate of this number including a count of all the sections of the vacuole).

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[1] The cells were observed in these species by Professor Ulric Dahlgren.

[2] With the exception of three figures especially referred to as *text-figures*, all figure references apply to the plates at the end of this paper.

Other vacuoles may show fewer large granules, or even a single very large one. It thus appears that the amount of this matter is fairly uniform in each vacuole. It is apparent also that in the fresh state the substance of the granules is soft and under a surface tension that permits of a union of two or more granules when the precipitate in which they lie permits of a close enough contact. This precipitate changes from the loose, fine reticulum of the full-sized vacuole to a denser and firmer mass which often holds the smaller granules in groups at the time the vacuole breaks and discharges its contents.

It is thus evident that these cells present an appearance greatly different from that of any nerve-cells known. A nerve-cell usually possesses a large round nucleus, a definite plasmosome, and a relatively small amount of chromatin material. Each of these cells, on the other hand, possesses an enormous nucleus of the irregular branching type, no definite plasmosome, and a large amount of chromatin. In the cytoplasm of nerve-cells chromophilic substance in the form of Nissl granules can usually be seen; in the cytoplasm of these cells no Nissl granules were distinguishable. No neurofibrils could be detected, though several special nerve stains were used. Moreover, the general staining reactions serve to bring out the fact that there is a definite chemical difference in the composition of these cells as compared with that of the neighboring nerve-cells.

Nerve-cells in the spinal cord of fishes are usually located approximately opposite or anterior to the structures which they innervate. These cells, as has already been pointed out, are present only in the caudal portion of the spinal cord from the level of the sixty-fourth vertebra to the tip of the tail. If they were nerve-cells, then we should expect to find the structures which they innervate in this region of the tail. But no special structures of any kind are present here, and here only. The electric organs, it is true, are situated in the tail, but they extend as far anteriorly as the thirty-third vertebra. The whole anterior half of the electric organs is anterior to the region of these cells. Dissection of the nervous system in the tail of the skate shows that the electroplaxes of the electric organs are supplied with nerves which are branches of spinal nerves, and these spinal nerves leave the spinal cord slightly anterior to the electroplaxes which they innervate. This would indicate that the electric motor nerve-cells in the spinal cord should be found approximately opposite to the electric organs. It is very improbable, then, that these large cells which are located opposite the posterior half only of the electric organs could be the electric motor nerve-centers. My observations lead me to agree with Ewart that the true electric motor nerve-cells are cells which resemble the ordinary muscle motor nerve-cells, except that they are larger.

HISTOGENESIS.

A few skate embryos of the species *Raia punctata* were collected and sectioned by Dahlgren at the Zoological Station at Naples. This material was turned over to the writer. The embryos were in a sufficient number of stages for the origin and development of the large, remarkable cells in question to be worked out. Stages were traced that showed them being differentiated from the same cells that also give rise to the motor nerve-cells of the anterior horn.

A typical series of early developmental stages was found in an embryo 7.3 cm. in length. The yolk-sac of this embryo was still fairly large. Sections taken at the level of the first tail-fin show how these cells become differentiated from the ordinary type of nerve-cell (figs. 9 to 16). There is first an increase in the amount and number of chromatin granules. The nucleus loses its spherical shape and becomes elongated (figs. 10, 11, 12). At a slightly later stage the nucleus becomes lobular as a result of unequal and localized growth (figs. 13, 14). The chromatin granules become more numerous. The first six figures of this plate have been drawn from cells from a single skate embryo. It is evident that all of the cells are not in the same stage of development. Some have hardly begun to show any differentiation at all, while others close to them have become well started on the growth and development that leads to the peculiar cells of the adult. Figure 17 is a drawing of a well-differentiated nerve-cell taken from the same embryo. It will be seen that this nerve-cell has already developed most of the typical nerve-cell structures, so that it is much like the nerve-cells of the adult. However, other nerve-cells are present which are less far along in their development.

Figures 15 and 16 are cells from the spinal cord of an embryo 22 cm. in length. The nucleus has become much larger and more lobular and the chromatin granules have also multiplied. The later development of the nucleus is merely a continuation of the process of unequal and localized growth, until finally it assumes the highly irregular, branching form that it has in the adult skate. The size of the cell-body as well as the size of the nucleus increases enormously, so that in sections of the spinal cord taken well down toward the tip of the tail this cell is one of the most conspicuous things in the field.

COMPARATIVE STUDY.

Several other kinds of fishes have been examined to see whether or not these cells are peculiar to the skate only. Cells resembling them more or less closely in appearance have been found in the dog-fish, shark, sting-ray, torpedo, goose-fish, gar-pike, and sword-fish; not, however, in *Fundulus*. It is not intended to give here a complete description of the morphology of these cells in each of the animals men-

tioned. However, a few points of interest about some of these forms will be taken up.

In all cases the cells are present in the caudal portion of the spinal cord, and they are even more restricted in anterior-posterior distribution than in the case of the skate. For example, in the dog-fish, *Mustelus*, they occur only from the level of the second dorsal fin (the caudal-most one) to the tip of the tail. In *Torpedo* they are present only in the region of the two little tail-fins. In the sting-ray and goose-fish the anterior limit was not determined exactly, but in the sting-ray the cells were found in the whip-like prolongation of the tail, and in the goose-fish they are about 2 inches from the caudal end of the spinal cord.

In the dog-fish, shark, torpedo, and sting-ray the cells are very much the same in general appearance as in the skate, but are much smaller, only about one-half as great in diameter as that of the skate in the case of the dog-fish. Indeed, the cells of the dog-fish almost exactly resemble cells of half-grown skates. One of the largest of these dog-fish cells is shown in figure 18. In the sting-ray the cells are smaller than in the dog-fish. The nuclei are somewhat different. They are not so highly lobular, but are concentrated into a more spherical mass. Several distinct nuclear membranes may often be made out, as if the nucleus had been formed from a union of several nuclei which had not quite completely fused. No cell vacuolation or granular material has been observed in either the dog-fish, torpedo, shark, or sting-ray.

The cells of the goose-fish, however, are the most interesting for this study. They differ greatly from all of the others. A photograph of one rather highly magnified is shown in figure 4. It resembles a nerve-cell in many respects. The nucleus is lobular and amœboid in shape, but much less so than in the case of the other fishes examined. Furthermore, it usually contains one or more plasmosomes, the first of these to be seen in any of these large and peculiar cells. The cell represented in the figure has three plasmosomes, one for each lobe of the nucleus. Only one plasmosome was in the particular section photographed, the other two being in adjacent sections. In an iron-hematoxylin van Gieson preparation these plasmosomes stain red with the acid fuchsin. The large chromatic granules characteristic of the nucleus in homologous cells of other fishes are not present. Instead, the chromatin is very finely granular and is scattered throughout on the reticulum that extends to all parts of the nucleus. The cytoplasm gives off processes which look as if they might contain some nerve-fibrils. No special nerve-stains have yet been tried. No Nissl granules have been made out. Occasionally the cytoplasm shows some vacuolation—very slight, however, compared with that shown by the cells of the skate. No granules are present. The cells do not seem to be limited strictly to the anterior horn, but in some cases are found very close to the outer membranes of the spinal cord. Moreover, in some cases as many as five or six of these cells were present in the same section.

It should be noted that the goose-fish is a teleost, whereas the skate, dog-fish, shark, torpedo, and sting-ray are elasmobranchs. Differences in structure should therefore be expected. It is possible, however, that these cells of the goose-fish are not homologous with the cells of the other fishes, although the fact that they are found in the posterior end of the spinal cord, just as are the other cells, makes it probable that they are homologues. At any rate, they certainly appear to represent a transitional stage between a nerve-cell and the highly differentiated cell of the skate. The evidence gathered from the study of the histogenesis of the cell of the skate indicated that the cell might be regarded as a transformed nerve-cell. The morphology of these cells of the goose-fish, because of their resemblance to nerve-cells, lends support to this view.

GLAND-CELL HYPOTHESIS.

Assuming, then, that these large, peculiar cells of the skate are transformed nerve-tissue, the question at once arises as to what their present function is. The cytoplasm of the cells seems to undergo vacuolation and partial liquefaction, with the production of some precipitate and granular material. This, of course, suggests glandular activity. The granules may represent some specific secretion that is being produced by the cells. The granules, after they have been manufactured and discharged, are not at once absorbed by the blood, but seem to persist in the tissues for some time. Most of them make their way down past the central canal toward the ventral side of the spinal cord. It is not known what causes most of the granules to gather in this region. Perhaps gravity pulls them in this ventral direction, or the movements of the neurolymph. Just ventral to the central canal a network of blood-vessels may often be seen. Here most of the granules are probably absorbed.

The whole process seems to indicate that these large cells are gland-cells of internal secretion. That such glands should be located in the anterior horn of the spinal cord seems to be remarkable; yet we know that other parts of the central nervous system have become profoundly modified. At various points in the brains of vertebrates are found places where the wall has been invaginated into long branching and anastomosing tubes of simple epithelium showing glandular activity. Blood-vessels have followed these tubes in and occupy their centers so that only a single layer of epithelium separates blood and brain fluids. Through this layer of epithelium substances are probably removed from, or passed into, the brain-cavity fluids. These structures are choroid plexuses.

The pituitary body is another well-known example of the way in which nerve-tissue may become modified. As this is also one of the endocrine organs, or glands of internal secretion, its structure is of

special interest for comparison with these large cells of the skate. The following brief but excellent description has been taken, with a few omissions and modifications, from "The Endocrine Organs," by E. A. Schaefer:

Like most of the other special endocrine organs, it is found with very few exceptions in all vertebrates. It is connected with the floor of the third ventricle by a short hollow stalk, the infundibulum. This stalk is composed of nervous tissue and expands into the interior of the gland into what is known as the pars nervosa, which when examined by appropriate methods is found to be mainly composed of neuroglia fibers and cells. In some animals the cavity of the infundibulum with its lining of ependyma is prolonged in the form of a blind canal far into the pars nervosa, but in man this canal has become obliterated, although it existed in the early embryo.

In front of and partly surrounding the pars nervosa the organ is formed of a mass of epithelial cells granular in appearance. This constitutes the pars anteriosa seu glandularis. It is highly vascular. In the middle of the organ the pars anteriosa is separated from the pars nervosa by a cleft lined by columnar, cubical, or flattened epithelial cells and filled with a glairy fluid.

The pars intermedia is a layer of epithelial tissue lying between the pars nervosa and this intraglandular cleft, not so granular and vascular.

Pars anteriosa. The cells are (1) clear, non-staining (chromophobe), and (2) granular, staining (chromophil); and the granular cells are again divisible into oxyphil and basiphil, the oxyphil cells being by far the most numerous. In some animals, notably elasmobranch fishes such as the skate, all of the cells of the pars anterior are set like a columnar epithelium around blood sinuses. Most of the cells also contain numerous fine fatty globules. There is a small amount of reticular connective tissue between the cells. A few nerves have been traced into the pars anterior from the pars nervosa. In some cases vesicles containing colloid may be present.

Pars intermedia. The cells contain fine neurophil granules. Often they surround well-defined vesicles containing oxyphil colloid. In addition to these colloid masses, some of the cells of the pars intermedia may often be seen in different stages of conversion into globular hyaline bodies, their protoplasm and nucleus becoming swollen; the latter may have become indistinct or have disappeared. Some of the globules thus produced are granular in character rather than hyaline. In both cases the cells ultimately break down, setting free the hyaline or granular substance. The pars intermedia is by no means everywhere marked off from the pars nervosa, for strands of the cells of the pars intermedia may extend a variable distance between the fibers of the pars nervosa. The hyaline and granular globules which have been derived from its cells also pass into the substance of the pars nervosa and are seen between its fibers; they can in fact be traced as far as the continuation of the third ventricle into the stalk. This fact was pointed out by Herring, who concluded that the hyaline and granular substances which are produced by conversion and breaking down of the cells of the pars intermedia form the secretion of this portion of the pituitary and that this secretion passes into the cerebro-spinal fluid. In confirmation of Herring's conclusion, evidence that the active principle of the posterior lobe of the pituitary is present in cerebro-spinal fluid has been obtained by Cushing and Goetsch, although their results have been traversed by Carlson. It has, however, been shown by Cow that intravenous administration of duodenal extract indubitably causes the appearance of the pituitary autacoids in the cerebro-spinal fluid, a fact which makes it evident that they must normally be passed to some degree into that fluid.

The hyaline globules are greatly increased as the result of thyroidectomy (Herring). Cushing states that this increase also follows extirpation of the pancreas and that it occurs as the result of section of the infundibular stalk.

Pars nervosa. This is formed almost entirely of neuroglia fibers with neuroglia cells scattered amongst them. Many of the fibers arise from these cells, others from the ependyma cells of the infundibulum and of its extension into the gland. Between the neuroglia fibers, especially in the neighborhood of the stalk, but also in other situations, is to be seen the hyaline and granular material already referred to; sometimes in the form of swollen cells, such as have been described in connection with the pars intermedia, sometimes as amorphous masses; these masses are traceable to the infundibulum, where they may be seen passing through the ependyma into the cavity of the ventricle. There can be little doubt that the physiological activity of extracts of the pars nervosa is connected with the presence of this substance within it, since extracts of ordinary nervous and neuroglia substance have not the same action. Some authors have described nerve-cells within the pars nervosa, but according to Herring these do not occur, and there are very few nerve-fibers. The pars nervosa is the least vascular portion of the pituitary, its blood-vessels being comparatively few in number.

Besides the cases of the pituitary body and the choroid plexuses we may mention the pineal gland and the adrenal bodies as other examples of original nerve-tissue that have become modified to form secretory tissue. The pineal body originates as an evagination of the dorsal wall of the diencephalon. It is composed of epithelial-like cells, trabeculæ, blood-vessels, many neuroglia cells and fibers, and cysts. There are no true nervous elements present. In function it is closely related to the sexual organs. Tumors involving the pineal body are often accompanied by very early development of sexual maturity with all the secondary sexual characters. Feeding pineal glands to very young chicks or guinea-pigs accelerates their development.

The suprarenal capsules consist of two parts, cortex and medulla, or, from a broader comparative view, interrenal and adrenal tissues. These parts are morphologically distinct, being developed from entirely different embryonic anlagen. The cortex is formed from mesoderm cells of the genital ridge. The medulla arises from cells which belong to the same neuroblast masses that give rise to the nerve-cells of the sympathetic ganglia. In fishes these two parts remain separate, but in all the higher vertebrates they are united into one organ anatomically. In mammals the medulla is centrally located, with the cortex inclosing it. The cells of the medulla are characterized by the brown color which results after treatment with chromic acid and its salts (chromaphil reaction). According to Moore, this reaction is due to the presence of adrenaline. The cells are arranged in irregular anastomosing columns with large blood-spaces between them. Chromophil granules are present which undoubtedly find their way into the blood-spaces directly. Besides the chromophil granules, other coarser ones are found, soluble in water and alcohol, but not in ether, and staining with difficulty. Lipoid and pigment granules may also occur. The tissue is very highly

vascular and richly supplied with nerves. Groups of sympathetic nerve-cells are occasionally found both in the medulla and in the deeper parts of the cortex. The substance adrenaline is secreted by the cells of the medulla. It acts as a powerful local styptic, raises blood pressure, and stimulates the heart. Injected subcutaneously it causes relaxation of muscles of the bronchioles; injected intravenously it causes glycosuria; in minute doses it causes checking of the rhythmic peristalsis of the intestine. It causes contraction of the uterus (non-pregnant) of the cat. In general, it acts especially on all functions innervated by the sympathetic system.

It was stated above that the cells of the medulla which secreted adrenaline were characterized by the chromophil reaction when treated with chromic acid. It is interesting to note that chromophil cells have been found in almost all vertebrates, even in *Petromyzon;* also in the mantle of the mollusk *Purpura* and in ganglion nerve-cells of annelids and crustaceans. The function of the chromophil cells always appears to be the same.

It was thought that the large cells of the skate spinal cord might be of this nature and that their sceretion might possibly be something allied to adrenaline. Accordingly the following experiments were tried:

Pieces of the spinal cord of *Raia lævis* were placed in Müller's fluid for two months. Upon sectioning, it was found that these large cells did not give a decided chromophil reaction.

Spaeth has shown that adrenaline will cause contraction of chromatophores, and that this reaction will be produced even when the adrenaline is diluted with water as much as 1 : 1,000,000. Fresh spinal cords from *Raia ocellata* were ground up and a sea-water extract prepared. Scales of *Fundulus* showing well-expanded chromatophores were immersed in this extract. The chromatophores were watched under the microscope. No contraction occurred.

Vulpian showed that adrenaline was readily oxidized by various reagents, giving characteristic color reactions with ferric chloride, chlorine water, and caustic alkalies. Addition of a sea-water extract of skate spinal cord to each of these caused no color changes.

Attempts were also made to discover the effects upon the blood pressure of the skate of an extract of the region of the spinal cord containing these large cells, but owing to the difficult technic involved in obtaining blood-pressure records from the skate no satisfactory results were obtained. The experiments given above are sufficient, however, to indicate strongly that these cells of the skate produce nothing that is allied to adrenaline.

Whatever the nature of the granules may be, their presence, together with the vacuoles and precipitate in the cells, fits in well with the hypothesis that these cells are glands of internal secretion. Vacuolation and partial liquefaction of the cell, followed by the formation, growth,

and discharge of granular material, are familiar steps in the process of secretion. This activity is undoubtedly aided greatly by the large, irregular nucleus. It is evident that a lobular, branching nucleus has a much greater surface-area than a spherical nucleus of equal volume. Interaction of nuclear and cytoplasmic materials is thus facilitated. Irregular branching nuclei, moreover, are known to occur in gland-cells, for example, in the spinning glands of the caterpillar. These cells in the skates are also in very close relation to the blood-stream. Wax reconstructions of the capillaries near some of the cells have been made, which show that one of the cells is usually touched by four or five capillaries (fig. 2). Indeed, I have found several cases in which a capillary for a part of its course was entirely surrounded by the cytoplasm of one of the cells. Such an intimate relation of the cells to the blood-vessels makes it favorable for them to extract from the blood the raw materials that they require for their activities and also for them to give back their specific secretion. Furthermore, the lack of a definite cell membrane, and the consequent loose integration of the cell cytoplasm, is favorable to glandular activity.

PHAGOCYTE HYPOTHESIS AND VITAL STAINING EXPERIMENTS.

E. V. Cowdry called the attention of the writer to the morphological resemblance of these cells of the skate spinal cord to the giant cells of the miliary tubercle. He suggested that instead of their being gland-cells they might in reality be phagocytes; that the granules instead of being secreted were being ingested. This hypothesis has some evidence in its favor and deserves careful consideration.

The giant tubercle cells have been shown to be pure colonies of endothelial macrophages. The term "macrophage" has been used by Evans to designate a certain class of cell which reacts in a definite way toward fine particles. He finds that when trypan blue, or certain other dyes of the benzidine series, is injected into living animals, the dye is taken up by certain groups of cells, the macrophages. These macrophages are abundantly present throughout the body, and line the blood-current itself in the liver, bone marrow, and spleen. Endothelium in most parts of the body may become phagocytic. Even in the central nervous system, which is usually unaffected even in animals whose tissues take an intense vital stain, the glial cells have been found to awaken in the case of lesions and to transform themselves into macrophages; and the endothelium of neural vessels may be similarly affected. The macrophages handle fats and fat-like bodies and the blood and bile pigments. Evans states:

"We may define macrophages as mononuclear cells wherever they may be, lining vascular channels, resident in connective tissue, or entirely free, whose protoplasm constitutes a physical system characterized above all by its response to finely particulate matter. In case of particles of ordinary micro-

scopic size, this behavior (phagocytosis) is shared with the polymorphonuclear ele nents of the blood. But towards ultramicroscopic particles the macrophages specifically drink them in."

By making use of this specific reaction, Evans, Bowman, and Winternitz have made an experimental study of the histogenesis of the miliary tubercle in vitally stained rabbits. They found that the giant tubercle cell is produced by a fusion of many endothelial cells, the stimulus of the tubercle bacilli being enough to cause endothelial cells to separate from the vessel-wall, form a syncytium, and begin their role of phagocytosis of the bacilli.

Might not the large, irregular cells of the skate have arisen in response to some stimulus, perhaps the stimulus caused by the presence of the granules, just as in response to the presence of the tubercle bacilli the giant tubercle cells arise? Morphologically the cells of the skate may be compared to the giant tubercle cells in several particulars. Both have a large amount of nuclear material, and the nucleus of the skate cells is often of such a distributed character that it may have arisen from more than one original cell, at least in some cases. As has been pointed out, the cytoplasm of the skate cells is occasionally distorted with vacuoles, precipitate, and granules. These vacuoles and precipitate might well be interpreted as granules in the process of ingestion and liquefaction. The fact that there is no definite cell-membrane would also make it easy for ingestion to occur. The conditions of surface tension seem to be like those of the macrophages. There is, moreover, a large blood-supply to these cells, a condition which would fit in well with a function of phagocytosis.

There is, furthermore, in the skate some evidence in favor of cell movement. The cell is quite variable in its position in the anterior horn. Sometimes the bulk of the cell is close to the central canal, with a long process reaching almost to the ventral artery. In other cases the cell is located much nearer the lateral edge of the anterior horn, with processes that extend laterally almost to the membranes of the spinal cord. The long processes might be regarded as pseudopodia capable of a very limited amount of motion. Figure 24, taken from a skate stimulated electrically, shows how the long medial ventral process has crowded and pushed aside the central canal cells so that Reissner's fiber, which normally is located in the middle of the central canal, has become entirely surrounded by the cell cytoplasm at one point.

Another remarkable condition is occasionally met with in a few cases. Sometimes a part of a process, together with some of the nuclear material, is found inside the central canal (fig. 42). It is difficult to explain how this state of affairs is brought about. It seems certain that the cell processes must be capable of at least a limited amount of movement. In this same figure a study of the appearance of the nucleus and even of the chromatic granules indicates that a probable

movement of a part of the cell-mass in a ventral direction has recently taken place. The chromatin granules are drawn out in threads in the direction of the medial ventral process and the whole aspect of the nucleus suggests that the cell has moved ventrally.

It should be kept in mind that the cells of the skate are found from embryonic stages on up through adult life. The tubercle cells, on the other hand, are of very recent origin, *i. e.*, they originate shortly after the tubercle bacilli become present. This may be one way of accounting for the difference in the appearance of the nuclei of the two kinds of cells. With longer duration the nuclei of the tubercle cells might fuse and come to resemble more and more the single branching nucleus of the skate cells.

The morphological similarities, therefore, of the giant tubercle cells and of the large cells of the spinal cord of the skate are close enough to justify experimental work that might throw some light upon the question of whether or not the two are also physiologically similar.

Skate 60. A 1 per cent aqueous solution of trypan blue was injected intraperitoneally; 19 hours later a second injection was given, and 24 hours later a third was given. The skate died overnight and the spinal cord was fixed in 10 per cent formol. Sections showed that no trypan blue had been taken up by these large cells.

Skates 67, 68. These animals were given the same treatment described above. The cells showed no trace of the dye.

Skate 36. One injection was given and the animal killed at the end of 42 hours. At this time there could be no doubt as to the success of the injection, as the whole skate was colored blue, including the tail. No trypan blue was found to be taken up by the cells in question.

Skate 38. One injection was given and the animal killed at the end of 5 hours. The cells showed no trace of the dye.

Skate 74. The spinal cord was exposed in the region of these cells near the first little tail-fin. Trypan blue was injected into the vertebral canal and into the small spinal vein on the dorsal side of the cord. At the end of 30 minutes the spinal cord was fixed in 10 per cent formol. The dye had not entered any of the large cells.

Skate 138. Trypan blue was injected into the spinal vein which lies on the dorsal side of the spinal cord. The injection was made in front of the first little tail-fin. The skate was kept alive for 10 hours and then pieces of the spinal cord fixed and examined. The dye had not been taken up by any of the large cells.

Skate 139. The animal was treated in the same way as skate 138. Pieces of the spinal cord were taken at the end of 10 hours. The skate was then kept alive for 22 hours more and other pieces of spinal cord then fixed. In neither case did the large cells show any trace of the dye.

These experiments indicate that the large cells of the skate do not belong to the macrophage group of Evans. In no case in the eight animals used was the dye taken up by these cells. It is well known that the blood-stream of the central nervous system is rather isolated from the rest of the circulation, and the objection may be raised that the vital dye did not get into the blood-stream of the spinal cord. Some of the neural blood-vessels, however, showed traces of the dye, and in one case in particular endothelium of capillaries well toward the central portion of the spinal cord took the vital stain, showing that in this animal at least the dye had permeated the vessels of the cord.

A few experiments were tried using other vital stains, some being composed of particles not so finely divided as in the case of trypan blue. Injections were made of janus green, methylene blue, carmine, and neutral red. In no case did the large cells in the spinal cord show any traces of the dye.

The evidence from these experiments with vital dyes does not by any means prove conclusively that the cells may not have a phagocytic function. It is conceivable that they might ingest only the granules that are normally present in the spinal cord, the reaction being a more or less specific one, and reject the finely particulate matter of the vital dyes. This, however, is certainly not probable. At any rate, these results show that the cells are not of the same nature as the macrophage group of cells.

CHEMICAL NATURE OF THE GRANULES.

1. *Tests for fat.*—Pieces of spinal cord were fixed in acetic sublimate, absolute alcohol, Flemming's fluid, and Perenyi's fluid. Sections prepared from each of these fixations were immersed in (*a*) xylol and (*b*) ether. After 24 hours the sections were examined to see whether or not the granules had been dissolved by these fat solvents. They were present in all except the Perenyi material. (As was found out later, the granules are dissolved by strong nitric acid. Since Perenyi's fluid contains nitric acid, this fact probably accounts for the absence of the granules.) Treatment with osmic acid failed to blacken the granules. Pieces of the spinal cord fixed in 95 per cent alcohol were sectioned and treated with Scharlach R dissolved in acetone alcohol. The granules remained quite unstained, although the medullary nerve-sheaths stained a pinkish red. In fact, in all these fat tests the reactions of the granules could be very conveniently compared to the reactions of the fat of the medullary sheaths. In all cases the reactions of the two were quite different. It is evident, therefore, that the granules are not fatty or lipoid in character.

2. *Tests for protein.*—The granules gave a positive Millon reaction. This result, of course, indicates their protein nature. The biuret, xanthoproteic, and Adamkiewicz tests were tried, but it was found impossible to complete any of these because of dissolution of the granules by the reagents used.

3. *Tests for zymogen granules.*—Mathews has shown that zymogen granules are speedily dissolved out by water and alcohol; that they are not preserved by alcohol, sometimes not by sublimate, nor by acetic sublimate. These granules are not dissolved out by water or alcohol, and are preserved by alcohol and by acetic sublimate.

The granules are not easily soluble. Fresh pieces of spinal cord were placed in salt solutions of various strengths: 5, 10, 20, and 30 per cent NaCl. Some pieces were placed in ordinary sea-water, some

in chloroform, and some in ether. After 24 hours the pieces were prepared for microscopic study to see whether the granules had been dissolved. In all cases a normal amount of granular material was present. Sections of the spinal cord from alcohol fixation were treated with a few acids and alkalies. The granules were found to be dissolved by nitric acid—not, however, by potassium hydroxide, sulphuric acid, or weak hydrochloric acid.

ELECTRICAL STIMULATION OF THE SPINAL CORD.

Experimental work was now undertaken to test, if possible, the hypothesis that these large cells of the skate were gland-cells. If the granules were being secreted by the cells, it was considered probable that electrical stimulation of the spinal cord would be followed by an increase in the amount of granular material.

Method.—In all of the following experiments the spinal cord was laid bare slightly anterior to the first tail-fin. Platinum electrodes, which led off from an induction coil attached to a single dry battery, were applied directly to the spinal cord. After stimuli had been sent in for the desired length of time, pieces of spinal cord were taken; one slightly posterior to the point of stimulation, one 2 inches posterior to the point of stimulation, and one about 4 inches posterior to the point of stimulation. These pieces were fixed in Bouin's fluid. Sections were cut 7 to 8 microns thick and stained with iron hematoxylin and van Gieson's stain. Skates of the species *Raia ocellata* were used, those of about the same size being selected. The first series of skates was collected in August, the second series in May.

First Series of Skates.

Skate 40: Stimulated for 2 minutes, fixed immediately. The cytoplasm of some of the cells showed disintegration and liquefaction, especially at the periphery. A few granules were present.

Skate 83: Stimulated for 2 minutes, fixed immediately. Very small granules are present. Vacuoles are present outside the cell filled with secretion.

Skate 41: Stimulated for 2 minutes, fixed 5 minutes later. Very small granules are present.

Skate 84: Stimulated for 5 minutes, fixed immediately. Granules are not present in very large amounts. Chromatic granules are often drawn out in strings as if movement has occurred.

Skate 44: Stimulated for 5 minutes, fixed 10 minutes later. Many granules are present. The nuclear material is usually arranged around the periphery of the cell. In figure 24 is shown a case where the ependymal cells of the central canal have been pushed aside by one of the cell processes. Reissner's fiber, which is ordinarily inside the central canal, is here entirely inclosed by the cytoplasm of the cell process.

Skate 26: Stimulated for 5 minutes, fixed 25 minutes later. Many granules of various sizes present, often being arranged in lines.

Skate 85: Stimulated for 10 minutes, fixed immediately. Granules present in rather large amounts.

Skate 86: Stimulated for 10 minutes, fixed 10 minutes later. Many granules present.

Skate 48: Stimulated for 10 minutes, fixed 30 minutes later. Many granules present.

Skate 49: Stimulated for 20 minutes, fixed 15 minutes later. Many granules present.

Skate 50: Normal animal that had been kept in the same environment as the animals that were subjected to the electrical stimulation. Comparatively few granules present.

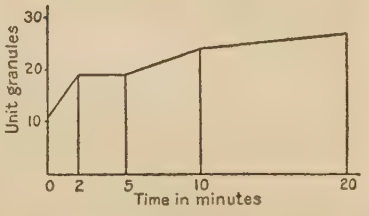
SECOND SERIES OF SKATES.

Skate 117: Stimulated for 2 minutes, fixed immediately.
Skate 118: Stimulated for 2 minutes, fixed immediately.
Skate 113: Stimulated for 5 minutes, fixed immediately.
Skate 114: Stimulated for 5 minutes, fixed immediately.
Skate 115: Stimulated for 10 minutes, fixed immediately.
Skate 116: Stimulated for 10 minutes, fixed immediately.
Skate 157: Stimulated for 10 minutes, fixed immediately.

Eleven other skates were taken as normal control animals and examined as to the amount of granular material present.

It appeared from superficial examination of the results of the experiments described above that electrical stimulation of the spinal cord caused an increase in the amount of granular material. Since, however, the amount of granular material present in any section varied greatly even in the same animal, it became desirable to adopt some method by which the average amount could be determined. The following method was used: A granule of a certain size was taken as a unit, and an estimation of the amount of granular material in terms of this unit was made in the case of each section. The results were averaged and the number of unit granules per section was computed.

From the 17 stimulated skates of both series of experiments an average of 17 unit granules per section was obtained. The 12 normal control animals showed an average of 10.7 unit granules. A definite increase in granular material, therefore, is correlated with stimulation of the spinal cord with electricity.



TEXT-FIGURE 2.—A curve showing relative amounts of granular secretion present in the spinal cord after stimulation with electricity for various lengths of time. The abscissæ represent the time of stimulation in minutes, the ordinates the number of unit granules per section. This curve is based upon 12 normal skates and 10 stimulated ones.

Text-figure 2 gives a graphical representation of the results obtained from the stimulation of the skates of the first series. The abscissæ represent the time of stimulation in minutes, the ordinates the amount of granular material in terms of unit granules. In skates stimulated for 2 minutes the average section contained about 19.4 unit granules. In skates stimulated for 5 minutes the average section contained 19.8 unit granules. In skates stimulated for 10 minutes the average section contained 24.4 unit granules. In those stimulated for 20 minutes the average section contained 27.1 unit granules. Increased granular secretion is here correlated also with the length of the time of stimulation.

PILOCARPINE STIMULATION OF THE SPINAL CORD.

Method.—The spinal cord was laid bare slightly anterior to the first tail-fin. The tip of the tail was then cut off about an inch from the posterior end and a solution of pilocarpine (0.1 per cent) injected into the vertebral canal. The posterior portion of the spinal cord was thus bathed in the pilocarpine solution, and it is probable that a certain amount of the drug entered the blood-stream by way of the spinal vein that lies directly upon the cord. From each of the skates treated in this way three pieces of the spinal cord were taken in the same way as was done in the experiments with electrical stimulation. These pieces were fixed in Bouin's fluid and the sections stained in iron hematoxylin and van Gieson's stain. The skates of the first series of experiments were collected in August, those of the second series in May.

First Series.

Skate 75: Stimulated for 5 minutes. Huge vacuoles present (fig. 20). Many granules are in evidence. In some cases the cell cytoplasm is filled with granules. The cells appear to be in a high state of activity.

Skate 76: Stimulated for 10 minutes. Many granules present. Especially noticeable is the close association of granules and cells. Figure 21 shows one of the large cells on one side of the central canal. Many granules are in this region, while on the other side of the central canal there is not a single granule, the reason seeming to be that there are none of the large cells near by.

Skate 77: Stimulated for 20 minutes. Some granules present, but not so many as in the case of the two preceding skates. No vacuolation of the cytoplasm of the cells could be noticed.

Second Series.

Skate 111: Stimulated for 2 minutes.
Skate 112: Stimulated for 2 minutes.
Skate 107: Stimulated for 5 minutes.
Skate 108: Stimulated for 5 minutes.
Skate 142: Stimulated for 5 minutes.
Skate 109: Stimulated for 10 minutes.
Skate 110: Stimulated for 10 minutes.
Skate 142: Stimulated for 10 minutes.

From an examination of the three skates of the first series of experiments it was concluded that pilocarpine stimulation of the spinal cord caused a very definite increase in the amount of granular material. The average amount of granular material per section in terms of unit granules was computed in the case of each skate, just as was done in the case of the skates stimulated with electricity. The skate stimulated for 5 minutes showed an average of 7.5 unit granules per section. The skate stimulated for 10 minutes averaged 28.3 unit granules per section. The skate stimulated for 20 minutes showed an average of 10.6. The average of the 12 normal control animals was 10.7. The first 2 skates of this series, therefore, show a remarkable increase in amount of granular material. The third skate is about normal. It was thought, however, that much of the apparent increase in granular material might be due merely to genetic variation; that in the animals used for this experiment there had chanced to be more than the average amount of granular material to start with. The second series of pilocarpine experiments showed that this was undoubtedly the case. In these

animals the average amount of granular material was much nearer the normal.

In all the animals stimulated by pilocarpine the average per section in terms of unit granules was 17.7, a definite increase over the normal average of 10.7. Plate 5 shows a few cells taken from the pilocarpine-stimulated skates. Figure 19 is a photograph of a cell showing an early stage in the secretion process. The central mass of cytoplasm is filled with vacuoles which contain a precipitate of some sort. This precipitate is probably formed largely from disintegrating cytoplasm, and in its staining reactions it behaves very much as the ordinary unchanged cytoplasm. The nuclear material appears to be placed at the extreme periphery of the cell. This is partly due, I believe, to the very recent dissolution and disintegration of some of the peripheral cell cytoplasm. A region of disintegration may be seen to the right of the figure, just outside the nuclear masses. Figure 20 illustrates the manner in which the cells are sometimes filled with enormous vacuoles. Five large ones and several smaller ones are to be seen in this case. Huge vacuoles are not very common in *Raia ocellata*. In *Raia punctata* they are found more often, even in the unstimulated animals. In figure 21 is a cell with a large amount of granular material near it. Probably a great deal of this had been formed, however, before the animal was treated with the pilocarpine. It is very interesting to note how the granules are all scattered through that half of the spinal cord in which the cell is located. None of them are to be seen to the right of the central canal. This close association of the granules and the cell is good evidence that the granules are produced by the cell as against their being phagocytosed by the cell.

ATROPINE STIMULATION OF THE SPINAL CORD.

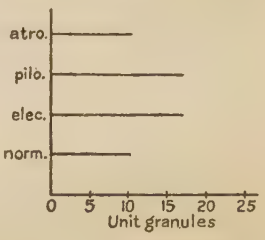
Atropine is an alkaloid whose effect upon gland-cells is directly the opposite of that of pilocarpine. It was, therefore, of importance to see what effect it would have upon these large cells of the skate and to compare these results with the results from the pilocarpine skates and also the normal control skates.

The method of administering the atropine was exactly the same as that used in the case of the skates stimulated with pilocarpine. In this series of experiments, 9 skates were used; 3 of these were treated with atropine for 5 minutes, 4 were treated with atropine for 10 minutes, and the other 2 were treated with the drug for 20 minutes. As before, the amount of granular material was computed in terms of unit granules per section.

The combined average of all 9 skates was found to be 10.6 unit granules per section. This was practically the same as found in the normal control animals. In other words, the atropine has no effect either in increasing or decreasing the amount of granular secretion.

DISCUSSION OF RESULTS FROM SPINAL-CORD STIMULATION.

The results from the preceding experiments may be summed up in the statement that electrical and pilocarpine stimulation cause definite increase in the amount of granular material, while treatment with atropine has no effect. Text-figure 3 indicates the relative amounts of granular material present in the normal animals and in those stimulated by each of the methods used.



TEXT-FIGURE 3.— Diagram showing the combined results from electrical, pilocarpine, and atropine stimulation, and how they compare with the normal average amount of granular material present in unstimulated skates. The abscissæ represent the average number of unit granules per section. The average number of unit granules per section from the normal and atropine stimulated animals is about the same. The average from the pilocarpine and electrically stimulated animals is much greater.

These results afford further evidence that the large cells are gland-cells. Pilocarpine is a specific gland stimulant, and electrical stimulation is ordinarily though not invariably followed by increased secretion. Atropine inhibits glandular activity. No marked decrease in the amount of granular material was noted in the skates stimulated by atropine, but this is believed to be due to the slowness with which the granules already present in the cord are absorbed by the blood-stream. It has already been pointed out that these granules are not very readily soluble, and in the spinal cord they evidently must persist for some time.

The number of animals used in these experiments is not very large: 17 for electrical stimulation, 11 for pilocarpine stimulation, 9 for atropine stimulation, and 12 as control animals. However, if the number of cells examined is taken into account, it will be seen that the results are based upon large numbers of cases. It is the cell and not the entire animal that is the real unit of variation in these experiments. The number of sections examined from the electrically stimulated skates was 6,716. As the gland-cells occur 1 about every 30 sections in the regions of the spinal cord examined, this number of sections would include 224 cells. The results from the skates stimulated by pilocarpine are based upon examination of 6,472 sections, or about 216 cells. The results from atropine-stimulated skates are based upon examination of 4,660 sections, or 155 cells, and the results from the control animals upon 7,144 sections, or 238 cells. The number of cases, therefore, is large enough to give good averages with no great deviations due to a few chance variations.

The method of estimating the volume of granular material in any section is admittedly not an exact one. Only approximately could it be calculated how many unit granules were equivalent to the amount of granular material present in any section. No better method, however, could be devised.

In addition to the increase of granular material demonstrated in the stimulated skates, a study of the sections was valuable in furnishing other evidence that the cells were of a glandular nature. The whole general appearance of the cells suggested this. Vacuoles inside and outside the cells were much more numerous than in normal skates. These vacuoles were usually filled with some precipitate or granular material or both. In some cases, however, they were entirely clear, filled with nothing but a fluid. Most noticeable of all, especially in the animals stimulated by electricity, was the presence of exceedingly fine granules; these appeared to originate in vacuoles, a large number of them being present in a single vacuole. The vacuoles were originally spherical or oval in shape, but in some cases were very irregular in outline. Figure 25, taken from a skate stimulated electrically for 2 minutes, is a typical example. The whole central part of the cell is filled with a huge irregular vacuole. At one end of this vacuole is some precipitate that does not stain with the iron hematoxylin. It has not yet been built up into granular secretion. The central part of the vacuole is filled both with lightly staining precipitate and with very fine granules arranged along this precipitate—granules which stain deeply with the hematoxylin. At the other end of this vacuole is a densely packed group of the fine, deeply staining granules. The conclusion is obvious. The lightly staining precipitate is gradually changed or gives rise to the fine granules as the process of secretion progresses. Down near the ventral edge of the spinal cord are two vacuoles filled with small granules that have become entirely separated from the cell which produced them, a very common occurrence. The smaller oval vacuole inside the cell shows, besides some very small granules, several larger ones, some of them evidently in process of fusing. In this vacuole the process of secretion has progressed farther than in the other vacuoles. This one cell, therefore, shows several stages in the process of secretion of granular material. It is typical of the stimulated skates that vacuoles filled with precipitate and fine granules should be present in much greater numbers than in the normal skates, and for that reason it is believed that they are a direct result of the stimulus applied. This evidence supports the theory that the cells are gland-cells.

Plate 8 (figs. 26 to 41) shows a series of vacuoles representing successive changes that occur in the process of secretion. All the figures except 36, 37, and 38 were taken from skates of the stimulation experiments, as those afforded the best examples. Figures 26 to 30 illustrate how the vacuoles may grow in size. Figures 29 to 32 show the appearance of a precipitate in the vacuole—precipitate which stains practically like the cell cytoplasm. Figures 33 to 36 show this precipitate becoming more thread-like, with fine granules arranged along the threads. In this precipitate appear granules which stain deeply with iron hematoxylin. Figure 36 is inserted here to show the definite

membrane that lines the vacuole. In this case the membrane is shown shrunken away from the cell cytoplasm so that it becomes plainly visible. A fully ripe vacuole (fig. 37) consists of deeply staining granules of various sizes. No precipitate is to be seen in these. Figure 38 shows a very large granule with other smaller granules in process of fusion with it. As it does not stain uniformly with the hematoxylin, it is probable that some of the material has not yet been completely built up into the ordinary granular secretion.

Before the ripe granules are fully formed the vacuoles may become separated from the cell that produced them. Figures 39, 40, and 41 represent vacuoles that have become separated from the cell at various stages in the process of granule formation. Figure 39 shows a vacuole with lightly staining precipitate only. In the vacuole of figure 40 are small granules with still some indication of the lightly staining precipitate. This vacuole was evidently separated from the mother cell later in its history than the preceding one. Figure 41 shows a vacuole with fully ripe granules, many of them in process of fusion to form larger ones. A remarkably fine group of vacuoles and granules outside the cells is shown in the photograph of figure 22. Some of these vacuoles show only the lightly staining precipitate, others show both the precipitate and granules, and still others only the fully formed granules. A number of granules are also present in the tissues of the spinal cord not inclosed by any vacuolar membrane. The ends of two of the large cells which gave rise to all of these vacuoles and granules may be seen.

It has been stated that the granules are probably absorbed by the blood, being dissolved as absorption takes place. In sections taken through the entire tail, however, granules were also found in the vascular connective tissue that is present lateral to each electric organ. These sections were taken about a half inch from the posterior tip of the tail. In sections farther anterior at the level of the first tail-fin no granules were seen. The significance of granules in this vascular connective tissue has not been determined.

Mention must also be made of a few morpohlogical variations or peculiarities that occur in connection with these cells. It has already been stated that vacuoles become separated from the cells. Sometimes a cell process, or part of a cell process, will break off entirely from the cell proper, losing all connection with it. Inasmuch as the cell is exceedingly variable in its position in the spinal cord, sometimes with a process that extends ventrally down past the central canal, reaching as far as the ventral spinal artery, and sometimes with a lateral process extending out to the enveloping membranes of the spinal cord, it is clear that these broken-off cell processes may be found in any region of the ventral half of the cord. If not enough nuclear material has also been broken off, the cytoplasm undergoes degeneration, taking on a peculiar dark-blue stain with iron hematoxylin. Many puzzling "dark

bodies" of the spinal cord in the region of these large cells are to be explained in this way.

Another striking peculiarity is the occasional occurrence of a portion of the cell inside the central canal. Figure 42 is a photograph of such a case. In this section the central canal is almost entirely filled with a part of one of these large gland-cells, both cytoplasmic and nuclear material being present, as well as some of the granular material. It was suggested to the writer by C. J. Herrick that these cells might have something to do with Reissner's fiber, perhaps representing the mechanism for renewing or regenerating Reissner's fiber in the posterior part of the cord. No real evidence has been found to support this hypothesis, however. None of the large cells are present in the anterior portion of the spinal cord or in the mid-brain, which is the anterior limit of Reissner's fiber. It would be expected that some of the cells would be present in these regions if this hypothesis were true. Furthermore, the granular secretion of the cells is rarely found in the central canal, so that it is improbable that it has any connection whatever with Reissner's fiber.

Another morphological peculiarity of the cells is the occasional presence in the nucleus of several plasmosome-like bodies. Several of these are indicated in the cell of figure 43. That these are not true plasmosomes is shown by their staining reactions. Their exact significance is unknown. E. G. Conklin has suggested that they may represent excretory products, formed perhaps as a result of unusual activity of the nucleus.

In the cytoplasm of this same cell are a few ependymal fibers. It has been observed in several other cases that ependymal fibers have in some way become incorporated in these large, irregular cells. As the cells are bounded by no definite cell-membrane they are rather loosely integrated, and assuming that they are capable of a limited amount of movement, it is easily conceivable how they might come to include some of the neighboring ependymal fibers.

EFFECT OF AMPUTATION OF THE TAIL.

The following experiments were made in order to discover whether these gland-cells with their secretion were of vital importance to the skate: The tails of 12 skates were cut off just anterior to the region containing the cells. The caudal artery was plugged with cotton to prevent excessive loss of blood, and the animals then placed in a large aquarium.[1] At the end of 48 hours the skates began to die. At the end of a week only 2 were still alive. The last one died at the end of 19 days. Most of the animals undoubtedly died as the result of injury caused by the operation. The fact that one of them lived for 19 days

[1]This large aquarium was the "dogfish pool" at the United States Bureau of Fisheries Station, Woods Hole, Mass.

shows that the animals can survive for some time at least without the secretion of these cells. More experiments must, however, be made before it can be said with certainty whether or not the cells are vital to the life of the skate.

REGENERATION.

One skate was collected which had at some time in its history lost the last 2 or 3 inches of its tail, only one little tail-fin being present instead of two. The skin had closed over the wound and grown together. Upon examining the end of the spinal cord it appeared that some regeneration had taken place, the last sixteenth of an inch of the cord being minus the usual line of black pigment along the mid-dorsal line. Upon sectioning this region one of the gland-cells was found, but it was very small, being no larger than an ordinary nerve-cell. A few small granules were also seen. It was concluded that this small cell represented an attempt at regeneration, probably having originated from the last normal cell—that is, the cell most posterior in position at the time of the injury.

FUTURE LINES OF INVESTIGATION.

Future investigation of this whole subject may be pursued very profitably in two directions. The first consists in a thorough comparative study. Undoubtedly the morphology of these cells in other forms of fishes, and perhaps in some of the higher vertebrates, will help to clear up their exact significance. It must be pointed out that the conclusion that the cells are gland-cells applies only to the skate. The appearance of the cells in the other fishes studied does not warrant the same conclusion. The cells of the goose-fish, particularly, look unlike gland-cells and more like nerve-cells. It may prove to be of great evolutionary significance to trace out the steps by which nervous tissue has become modified and transformed to glandular tissue, as illustrated by the comparative morphology of these cells. Another promising line of investigation is to be found in experiments with extracts of the granular secretion. Unlike the secretion of other endocrine organs, these granules are not readily soluble in water and alcohol, and it may not be easily possible to obtain a fairly pure extract. However, if a satisfactory method of getting this is worked out, the results following administration of the extract to animals should prove to be of interest. The writer hopes to pursue both of these lines of investigation.

SUMMARY.

1. In the posterior portion of the spinal cord of the skate there are present large, irregular cells of peculiar structure.

2. These cells are present in the anterior horn on each side of the central canal.

3. There are about 600 in all in an animal of the species *Raia ocellata*.

4. The nucleus of each cell is a lobular branching structure extending throughout the cell. The chromatin is scattered throughout the nucleus in the form of large granules. No definite plasmosome is present.

5. The cytoplasm of a resting cell is homogeneous. In active stages, however, the cytoplasm is characterized by the appearance of small vacuoles which often coalesce into larger ones. Secretion appears in these, and finally the contents of the vacuoles are discharged in the form of granules which persist in the tissues of the spinal cord for some time.

6. This granular material is protein in character. It is not easily soluble.

7. These cells develop from the same neuroblast tissue that gives rise to the muscle motor nerve-centers and electric motor nerve-centers.

8. Cells homologous to them have been found in the spinal cord of torpedo, sting-ray, dog-fish, shark, goose-fish, gar-pike, and sword-fish; not, however, in *Fundulus* nor in *Necturus*.

9. The following theories as to the nature of these cells have been considered: (1) That they are nerve-cells; (2) that they represent parasitic or pathological conditions; (3) that they are phagocytes; (4) that they are gland-cells of internal secretion. The evidence, morphological and experimental, indicates that the cells are gland-cells of internal secretion. The experimental evidence consists in increase in volume of granular material following electrical and pilocarpine stimulation of the spinal cord. No increase in volume of granular material follows atropine stimulation.

10. The exact function of the granular secretion of these gland-cells is unknown.

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EXPLANATION OF PLATES.

ant. h.. . . . anterior horn of gray matter.
cap.. . . . . capillary.
c. c.. . . . . central canal.
epen.. . . . . ependymal fibers.
gl. c.. . . . . large gland-cell.
gr.. . . . . granules secreted by large gland-cell.
men. pr.. . . menix primitiva.
nuc.. . . . . nucleus.
nv. c.. . . . . nerve-cell.
pl.. . . . . plasmosome.
post. h.. . . posterior horn of gray matter.
prec. d.. . . precipitate staining dark with iron hematoxylin.
prec. l.. . . precipitate staining light in sections stained with iron hematoxylin and van Gieson's fluid.
R. f.. . . . . Reissner's fiber.
sp. a.. . . . . ventral spinal artery.
sp. v.. . . . . dorsal spinal vein.
vac.. . . . . vacuole.
x.. . . . . unknown plasmosome-like bodies in nucleus.

Plate 1.

Fig. 1.—Cross-section of spinal cord of *Raia ocellata*. Two of the large gland-cells are present, one on each side of the central canal and close to it. In the anterior horn to the right is a nerve-cell, a comparison with which will bring out the large size of the gland-cells. Note the peripheral distribution of the nucleus of the cell to the right. Photograph ×146.

Fig. 2.—Photograph of a wax reconstruction of one of the large gland-cells of *Raia ocellata*, together with the capillary network about it. The white wax represents the cell, the dark wax the capillaries. Four capillaries may be seen. ×513.

Fig. 3.—Photograph of a wax reconstruction of the nucleus of the cell shown in figure 5 from *Raia lævis*. Note the irregular lobular structure and the large size. ×292.

Fig. 4.—Cell from spinal cord of the goose-fish, *Lophius piscatorius*, thought to be homologous to these cells of the skate. A well-defined plasmosome is present in one of the lobes of the irregular nucleus. Plasmosomes were also present in the other two lobes in the sections immediately preceding the one shown. Photograph ×1177.

Plate 2.

Fig. 5.—A single large cell from spinal cord of *Raia lævis*. Vacuoles and granules are present in the cytoplasm. Some of the discharged granules may be seen near the cell. The nucleus, apparently multiple, is in reality a single one. A reconstruction of this nucleus is shown in figure 3. ×1028.

Plate 3.

Fig. 6.—A cell from longitudinal section of spinal cord of *Raia punctata*, showing many small vacuoles in the central masses of the cytoplasm and one large one. Photograph ×734.

Fig. 7.—A cell from a longitudinal section of the spinal cord of *Raia punctata*, showing about 9 large vacuoles. The nuclear material is centrally located and the vacuoles are at the periphery of the cell. Photograph ×734.

Fig. 8.—A cell from a longitudinal section of spinal cord of *Raia lævis*, showing 2 vacuoles containing granules which are about to be discharged. Photograph ×531.

Plate 4.

Figs. 9, 10, 11, 12, 13, 14.—Cells from spinal cord of a *Raia punctata* embryo 7.9 cm. in length. Successive stages in the early development of the large cells, starting with the relatively undifferentiated cell of figure 9. Growth of cell and early lobulation of nucleus are well shown. ×1210.

Figs. 15, 16.—Cells from spinal cord of a half-grown skate, *Raia punctata*, 22 cm. in length. The cell has become much larger and the nucleus much more lobulated. ×1210.

Fig. 17.—A nerve-cell shown for comparison, taken from the spinal cord of a *Raia punctata* embryo 7.9 cm. in length. ×1210.

Fig. 18.—A cell from spinal cord of the dog-fish, thought to be homologous to these cells of the skate. ×1070.

Plate 5.

Fig. 19.—A cell from spinal cord of *Raia ocellata*, stimulated with pilocarpine for 5 minutes. The central portion of the cell is filled with vacuoles containing precipitate which stains much like the cell cytoplasm. The nuclear material is peripherially arranged. Photograph ×750.

Fig. 20.—A cell from spinal cord of *Raia ocellata* stimulated with pilocarpine for 5 minutes. Five large vacuoles and some smaller ones are shown in this section. Photograph ×531.

Fig. 21.—Cross-section of spinal cord of *Raia ocellata*, stimulated with pilocarpine for 10 minutes. One large gland-cell is present in the anterior horn on the left. Note the heavy, dark-staining granules, which are present only on the side of the spinal cord on which the cell is. Much more than the normal volume of granular material is present in this section. Photograph ×153.

Plate 6.

Fig. 22.—Longitudinal section of spinal cord of *Raia lævis*, showing vacuoles, precipitate, and granules outside the gland-cells in various stages of development. There are present in this section vacuoles precipitate, vacuoles with small and large granules, and granules of various sizes

that have been discharged by vacuoles and are now in the tissues of the cord. The end of a large gland-cell may be seen at each side of the figure. A much larger amount of granular material is normally present in *Raia lævis* than in *Raia ocellata*. Photograph ×438.

Fig. 23.—Cross-section of spinal cord of *Raia ocellata*, after stimulation with electricity for 10 minutes. Much more than the normal amount of granular material is present. The cell which produced this is not shown in this section. It was present, however, a few sections farther on. Photograph ×530.

Plate 7.

Fig. 24.—Cross-section of spinal cord of *Raia ocellata*, after stimulation with electricity for 5 minutes. The animal was killed 10 minutes later. One of the large gland-cells has crowded aside the central cells. Reissner's fiber, which is ordinarily inside the central canal, is here shown entirely inclosed by the cytoplasm of the cell. Granular secretion is also present in the tissues of the cord. Note also the peripheral distribution of the nuclear material of the cell. ×490.

Fig. 25.—Cross-section of spinal cord of *Raia ocellata* after stimulation with electricity for 2 minutes. A large gland-cell is present just dorsal to the central canal. A large, irregular vacuole is to be seen filled with precipitate, some of which is light-staining and some dark-staining. Down near the ventral edge of the cord are to be seen 2 vacuoles filled with dark-staining precipitate. These have also probably been produced by the cell. Another vacuole filled with mature granules is also present in the cyptoplasm of the cell. ×612.

Plate 8.

Figs. 26–41.—A series of vacuoles taken chiefly from skates stimulated with electricity or pilocarpine, illustrating the complete history of the vacuoles and granules.

Figs. 26–32.—These show the growth of the vacuoles which at first are filled with a clear fluid, later with precipitate which stains much like the cell cytoplasm. This precipitate later becomes thread-like and granular, staining at first lightly, then taking a deep iron-hematoxylin stain (figs. 33–35).

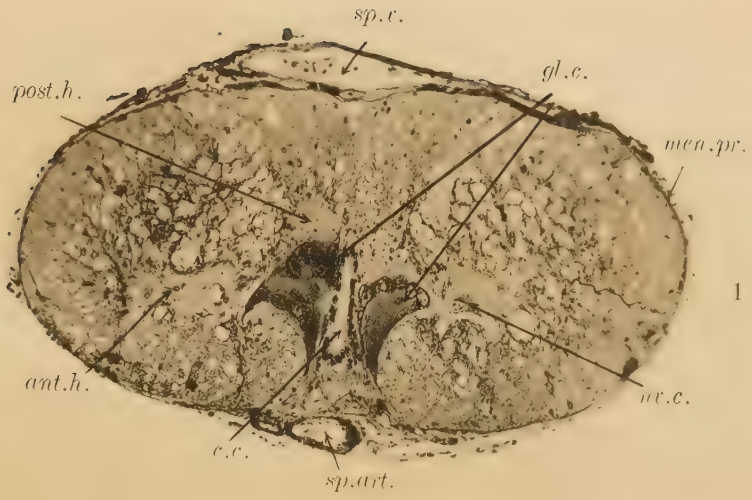
Figs. 36–38.—These are taken from *Raia lævis*. Figure 36 is here inserted to show the definite membrane that surrounds the vacuole. The cell cytoplasm has shrunk away from the vacuole, bringing the membrane plainly into view. Figure 37 represents a fully ripe vacuole containing granules about to be discharged. Figure 38 shows a single large granule and a few smaller ones. The large one has been formed by fusion of many smaller ones and, as can be seen from its staining reactions, the fusion is not entirely complete.

Figs. 39–41.—Vacuoles outside the cell. Figure 39 shows one that must have been isolated from the cell which produced it while it still contained the light-staining precipitate, before any ripe granules had formed. Figure 40 shows a vacuole that was isolated from the mother cell just as the light-staining precipitate was being replaced by very small dark-staining granular material. Figure 41 shows a vacuole containing ripe granules partially fused together. Although the vacuole is ripe the vacuolar membrane has not yet broken. ×1012.

Plate 9.

Fig. 42.—Cross-section of spinal cord of *Raia ocellata*, after stimulation with electricity for 2 minutes. A part of one of the large gland-cells has in some way entered the central canal and fills it up almost entirely in this section. Note also how the cell-body and even the chromatin granules of the nucleus have been drawn out ventrally, as if the cell had recently moved in this direction. Photograph ×530.

Fig. 43.—Cross-section of spinal cord of *Raia oecllata*, after stimulation with electricity for 2 minutes. In the nucleus may be seen several of the unknown plasmosome-like bodies. Note also the ependymal fibers apparently running through the cell cytoplasm. Photograph ×530.



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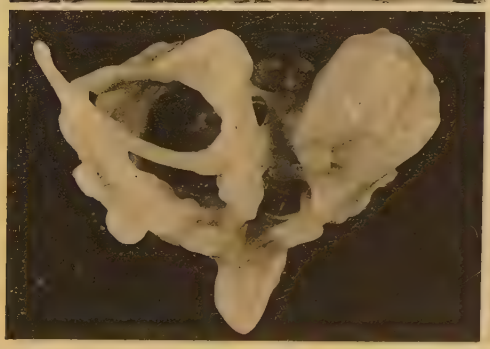

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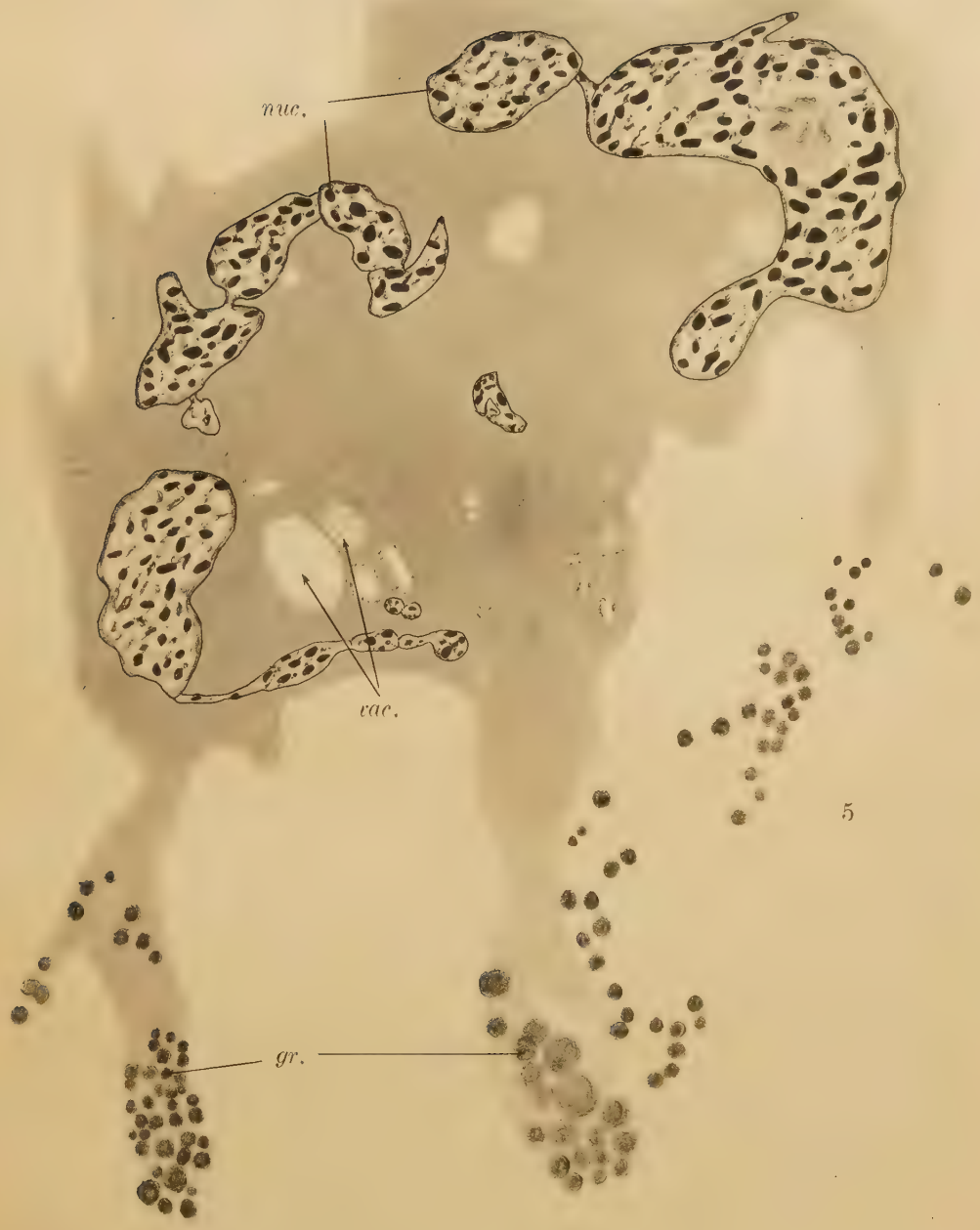
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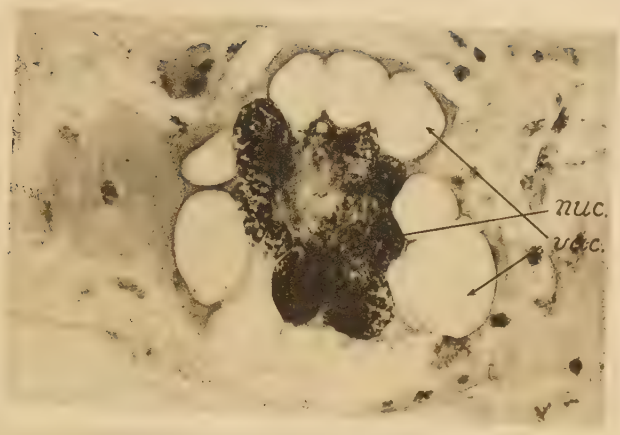

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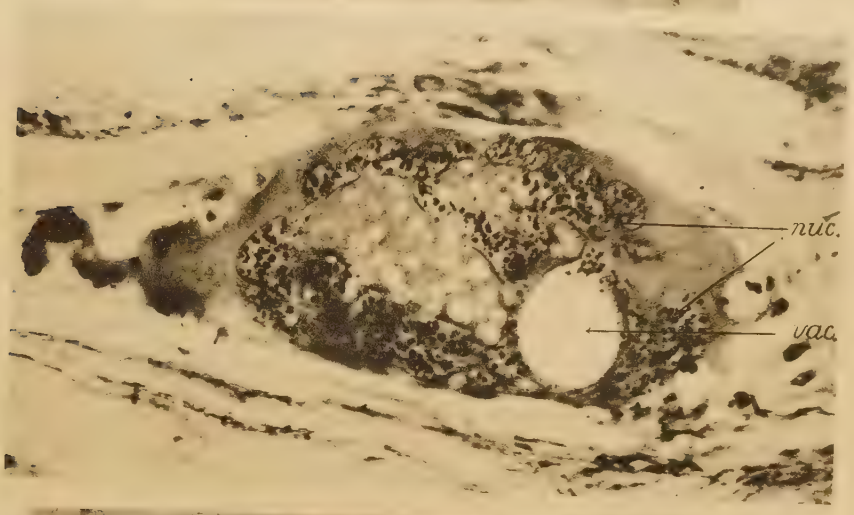

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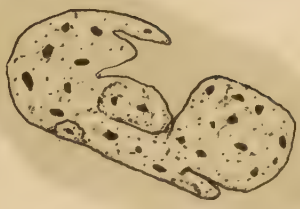

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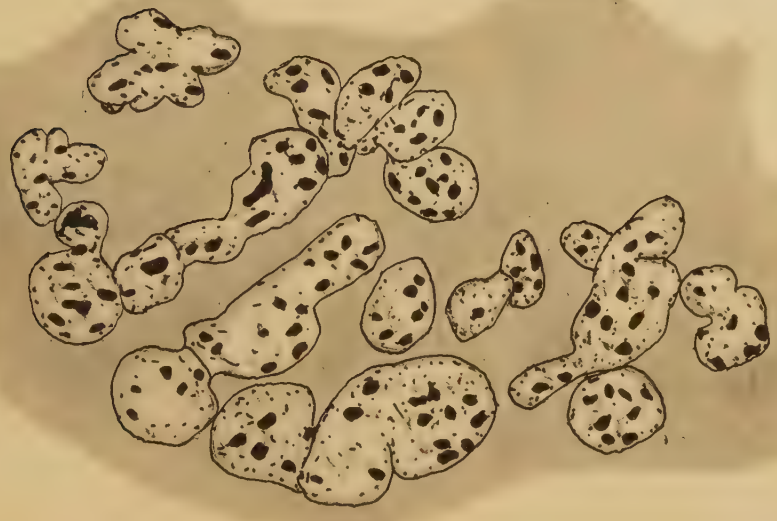

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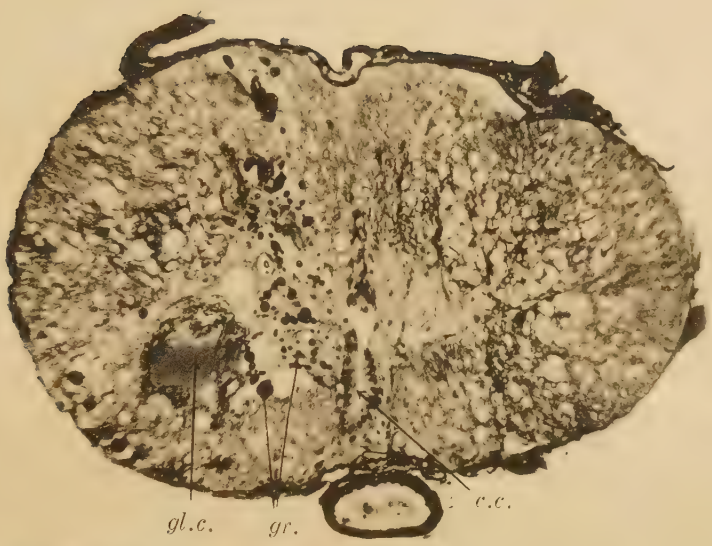

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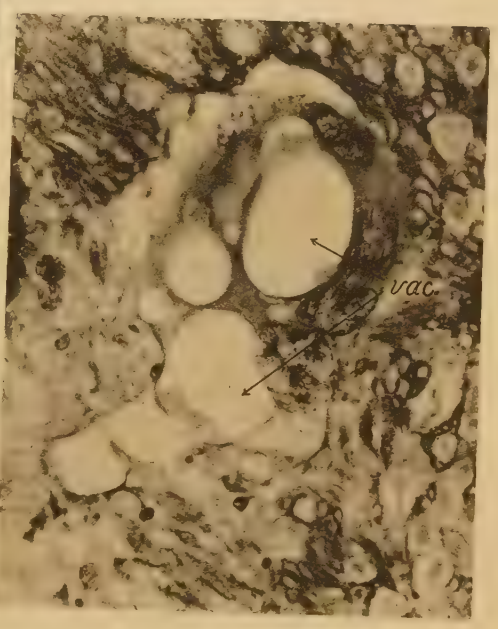

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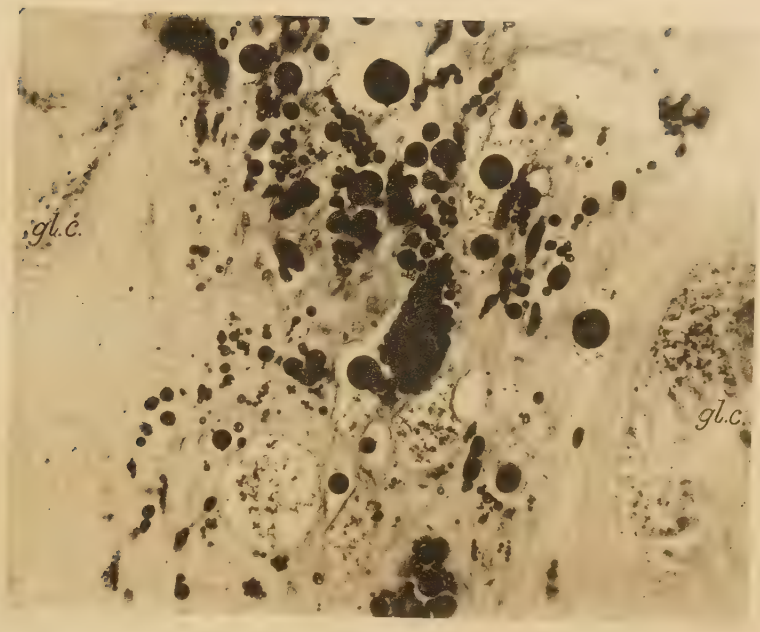

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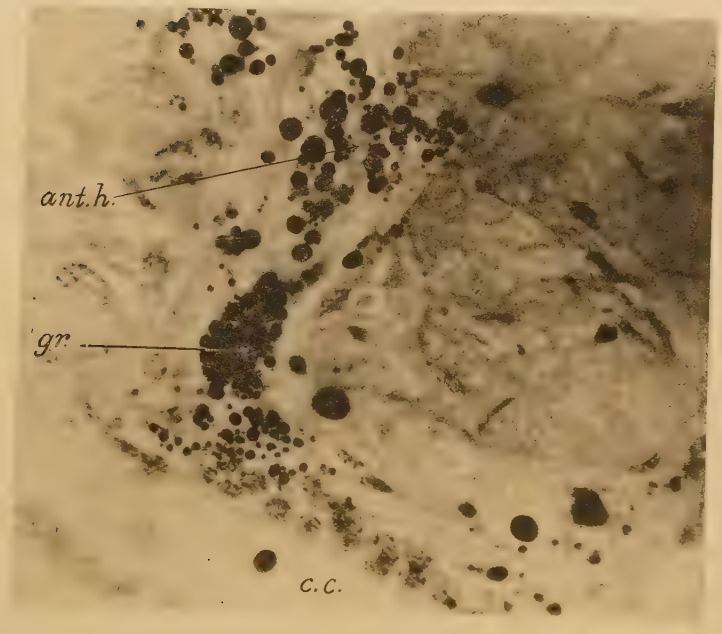

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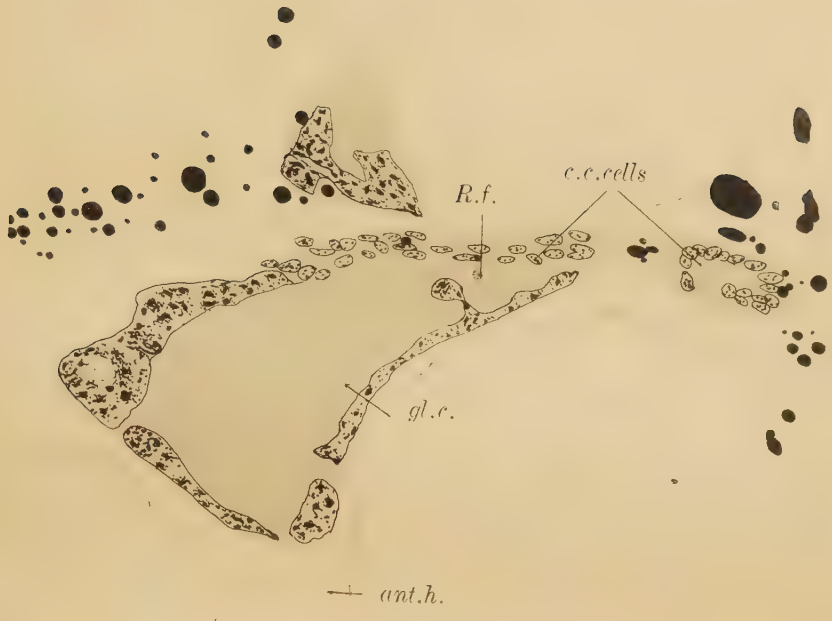

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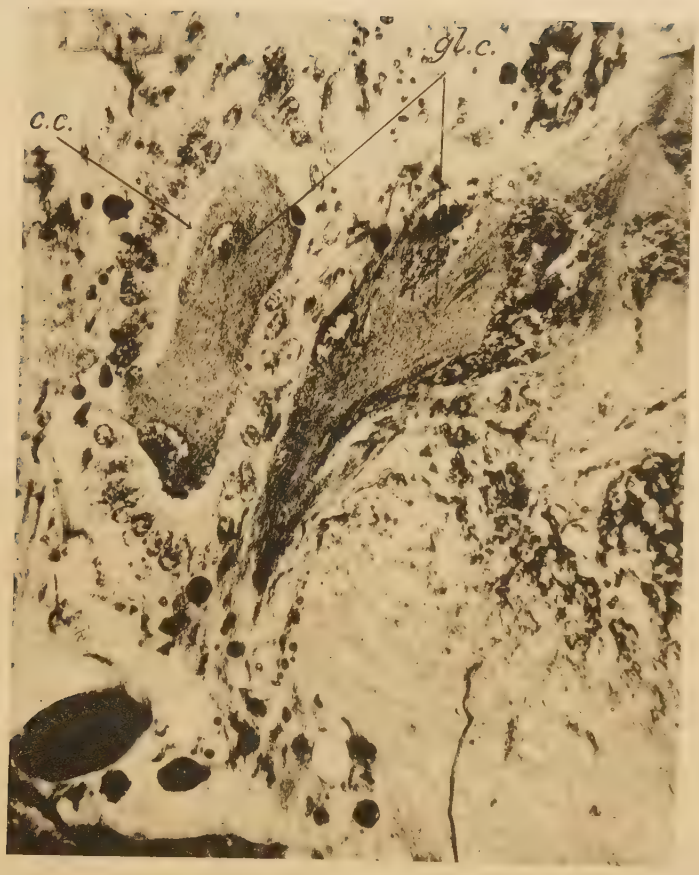

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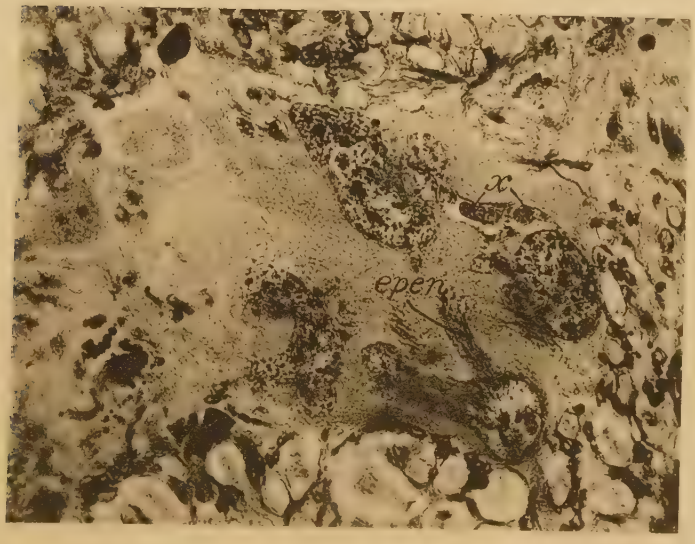

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II.

THE STRUCTURE AND EJACULATION OF THE SPERMATOPHORES OF OCTOPUS AMERICANA.

By GILMAN A. DREW,
Marine Biological Laboratory, Woods Hole.

Three Plates.

THE STRUCTURE AND EJACULATION OF THE SPERMATOPHORES OF OCTOPUS AMERICANA.

By Gilman A. Drew.

It was my privilege to spend several weeks in February and March 1912 at Montego Bay, Jamaica, and to enjoy the privileges of the Carnegie Marine Biological Laboratory established there that year. For several years I had been working at problems relating to the spermatophores of the squid and this gave me an opportunity to study the spermatophores of an octopod. I regret the delay in publishing the results, but the preserved material has kept in very good condition. Advantage was taken of a sojourn of several weeks at Tucson to complete the observations and drawings, and I greatly appreciate the courtesies extended to me by the staff of the Biological Department of the University of Arizona during that period.

Octopus is quite abundant in the waters near Montego Bay and as the animals are used as food they are collected by the fishermen and taken to the markets, where they are sold under the name of "sea-cats." Most of the material used was purchased directly from the fishermen and many of the specimens were alive and vigorous when received.

Fishermen frequently get these animals in their baited fish-traps, which somewhat resemble the lobster pots used in northern regions, and they sometimes drag them from crevices in the rock with sharp hooked wires. Immediately upon catching a specimen it is the usual practice to "turn the cap" to keep it quiet. This consists in catching hold of the edge of the mantle near the funnel and forcibly turning it wrong side out. In doing this the connections of the mantle are torn and the viscera are disarranged and usually mutilated. While this serves to "keep the animals quiet" it is not a desirable method for one who wants to study them. By increasing the price paid for unmutilated material it soon became possible to get all that were needed, so other methods of collecting did not have to be devised.

The spermatophores of these animals are very much larger and not so turgid as those of the squid. They are stored in a spindle-shaped spermatophoric sac that lies along one margin of the spermatophoric organ. Part of the spermatophoric sac extends between the spermataphoric organ and the almost globular testis. The testis is inclosed in a membranous capsule and this, together with the vas deferens, spermatophoric organ, and spermatophoric sac, is inclosed in another membranous capsule that adheres to the capsule of the testis but evidently has no communication with it. Through this outer capsule the sexual duct or penis protrudes a short distance.

The outer capsule may be cut and easily slips back, so that the spermatophoric sac, the spermatophoric organ, and the vas deferens are exposed, but all are held together (as by a mesentery) by the wall of the capsule that joins the testis capsule.

The inside of the wall of the spermatophoric sac is thrown into a large number of deep longitudinal folds, among which the spermatophores lie, with their aboral ends toward the penis. As many as 79 spermatophores have been taken from a single spermatophoric sac.

Spermatophores of cephalopods are mentioned often by zoological writers, but mostly as mere references. Descriptions and figures when given are usually incomplete or unsatisfactory and show plainly that not much careful study has been made by the authors.

Emile G. Racovitza (1894*c*) has given an excellent account of the structure and ejaculation of the spermatophore of *Rossia* and I have a rather more extended account of the spermatophores of the squid, *Loligo*, now in press (1919).

In describing the spermatophores of *Octopus* comparison will be made to the spermatophores of *Loligo* described in the paper to which reference has just been made and the same system of lettering for the figures will be used. Inasmuch as the octopus spermatophore is simpler than that of the squid and certain structures found in the latter are not present in it, the reader of this paper will find certain peculiarities in naming. In the spermatophores of *Octopus* there is no inner tunic, and if there is an outer membrane it is so thin that it has not been identified. Nevertheless the terms middle tunic and middle membrane have been retained, although they do not occupy medial positions in respect to other layers. Specimens of spermatophores removed from the spermatophoric sac and placed immediately in about 10 per cent formalin do not discharge and remain transparent and easily studied. Placing them directly into full strength formaldehyde does no harm, but the added strength is not needed. The membranes and tunics are hardened somewhat by this reagent and, when they are to be stained and mounted, somewhat better results can be had if they are not left many hours in the formalin. The chief difficulty is with wrinkling when the membranes are hardened, but this can be overcome by slow diffusion methods.

The most successful stain for most purposes has been Ehrlich's triacid. The stain may be diluted either with water or formalin solution, and it is usually better to use several times as much water as stain. The exact strength does not seem important, but with stronger solutions the spermatophores are stained much quicker. When removed from the stain the spermatophores are washed and placed in 10 per cent formalin for a few minutes and then mounted directly in glycerine jelly. If the membranes have hardened so that wrinkling

is likely to occur, they may be placed in a diffusion apparatus with glycerine over night and mounted in glycerine jelly the next day.

I have found a very simple diffusion method with glycerine is to place the spermatophores in a concave watch-glass filled with formalin. Place this in a shallow stender dish of about the diameter of the watch-glass, fill the space between the two dishes with glycerine and flood with formalin, so that the diffusion may take place over the edge of the watch-glass.

Specimens mounted in glycerine jelly will be clearer than those in formalin and may be studied at leisure. The stain fades slowly, but is sufficiently permanent to be effective some months.

STRUCTURE.

The spermatophores of *Octopus* differ in size with the size of the individuals from which they are taken. Large ones measure as much as 50 mm. in length, small ones may not be more than two-thirds as long. The spermatophores are slender and taper irregularly from the aboral to the oral end. The sperm mass (fig. 1 SM), which is white and opaque, lies in the aboral end of the spermatophore and occupies about one-third of its length. The ejaculatory apparatus occupies between one-third and one-half of the oral end of the spermatophore and is quite transparent.

Between these two portions and extending along the sides of the sperm mass and ejaculatory apparatus is a space occupied by liquid in which is a considerable mass of granular material (fig. 1 z). This material mixes readily with water and it may have an important osmotic property. The bulk of it occupies a position between the sperm mass and ejaculatory apparatus that corresponds to the position of the cement body in the squid, but it evidently has no cementing property and is not inclosed in a special capsule. No cement is needed in this spermatophore, as the sperm are not loaded by the ejaculating spermatophore into a sperm reservoir that has to be stuck into position, but are evidently introduced directly into the oviducts of the female. Whether there is any homology between these materials in the two forms is not clear. Their similar positions are significant but their functions are evidently entirely different.

The spermatophore is turgid and elastic but not nearly so much so as the spermatophore of the squid, which is so turgid that when bent it will, upon release, assume its original shape immediately. The squid spermatophore may even be picked up by one end with forceps and will stick straight out without appreciably bending. The octopus spermatophore may be bent into flowing curves on the bottom of a dish and remain as left, provided none of the curves are abrupt. It is nevertheless under considerable tension, for when the outer covering of a fresh spermatophore is cut, the contents are thrown from

the cut rapidly. The turgidity, as in the case of the squid spermatophore, is due to the tough elastic and stretched outer tunic.

The outer tunic (figs. 1 and 5 OT) is thin and nearly transparent, although faintly amber in color. It is not as colorless as that of the squid and is much thinner. Magnified as shown in figure 1, it is so thin that it is represented by a single line. The fact that, although the spermatophore of *Octopus* is much larger than that of the squid, the outer tunic is actually thinner, accounts for the difference in turgidity and that in ejaculation *Octopus* spermatophores are much slower than those of the squid.

The outer tunic forms the covering for the spermatophore to the oral extremity. The oral end is covered by the cap (figs. 1 and 2 c), which is thinner than the outer tunic, more transparent, and is evidently more affected by water.

Over the cap is thrown a broad cap-thread (CT) that adheres rather strongly to one side of the spermatophore and extends aborally for a distance considerably greater than the length of the ejaculatory apparatus. The other end of the cap thread seems always to be free from the spermatophore, but is folded well over the extremity of the cap, frequently back to the end of the ejaculatory apparatus, as a broad, striated bandage.

Pulling the cap thread seems to start ejaculation of the fresh spermatophore. How it is used to start normal ejaculation is not known, but it seems probable that the thread is pulled in some way. The spermatophore leaves the penis aboral end first and it seems reasonable to suppose it reverses ends when started down the groove of the hectocotylized arm. It is not known how it reaches this groove, but the groove has no connection with the penis and it seems probable that the spermatophore must pass through the funnel in being transferred to the groove. The mechanics of the transfer is not known, but it is probable that the thread is pulled during the process. A spermatophore placed in sea-water will usually ejaculate rather promptly even when the cap thread is not pulled intentionally. This may be due entirely to osmotic changes and dissolving effects of the water or it may be that the thread is always disturbed enough by removal from the spermatophoric sac to weaken the cap end.

Inside the aboral portion of the outer tunic, corresponding pretty closely with the length of the sperm mass, is a closely adhering membrane, the middle tunic (fig. 1 MT). This is not nearly as thick and conspicuous as the middle tunic of the spermatophore of the squid and does not show a granular structure. It does not line the extreme aboral end of the outer tunic and gradually thins out and disappears just beyond the oral end of the sperm mass.

The middle tunic evidently swells rapidly in water, but it does not have as great osmotic properties as the middle tunic of the spermato-

phore of the squid. The difference in the strength and stretch of the outer tunics and in the osmotic properties of the middle tunics is doubtless responsible for the difference in rapidity of ejaculation in the two forms. The whole process is usually complete in less than 10 seconds in the squid and may occupy from $1\frac{1}{2}$ to 3 minutes in *Octopus*.

The liquid that occupies the space between the sperm mass and the ejaculatory apparatus is continued along the sides of these two portions between them and the outer coverings, so there is no adhesion between them and the outer tunic or middle tunic except where the ejaculatory apparatus is permanently attached to the outer tunic at the oral extremity. The liquid that occupies this space is not so absolutely transparent as it is in the squid spermatophore, but is noticeably granular throughout. It is much more granular in the space between the sperm mass and ejaculatory apparatus, but nowhere is it entirely free from granules.

Along the sides of the sperm mass the space occupied by the liquid is always distinct (fig. 1 SL). Along the sides of the ejaculatory apparatus it is frequently hard to find except in the grooves between the loops of the spiral into which a portion of it is thrown (fig. 3 SL). Ejaculation shows, however, that there are no adhesions at any point and that the liquid serves as a lubricant and to transmit pressure during the act.

The sperm mass (fig. 1 SM) consists of a thread of sperm, mixed with a somewhat granular viscid secretion, that is wound into a cylindrical spiral. The separate loops, unlike those of the squid sperm mass, are distinct with the outlines rounded. The loops are not compressed against each other enough to flatten their adjacent sides very much and the mass is not inclosed in an inner tunic. That the inner tunic is completely absent in this form is indicated by the fact that when ejaculation begins the sperm thread begins to uncoil and straighten (fig. 16 SM). This would not be possible if the sperm mass were inclosed in a definite tunic, as is the case in the squid.

The sperm thread is not of entirely even diameter throughout and the coiling is not entirely regular, but the irregularities are only imperfections and have no functional significance.

The sperm mass is usually referred to by authors as the sperm rope. In using this term it should be borne in mind that unlike a rope it consists of but a single coiled strand.

The ejaculatory apparatus, while easily compared with that of the squid, differs in the absence of the inner tunic and outer membrane and in its end relationship, for this form has no cement body. It is perhaps questionable whether there is an outer membrane, but I have not been able to distinguish one. The middle membrane is thick, evidently very pliable, and (as in the case of the squid) is composed of many thin longitudinal layers that are presumably due to the

winding of a thin sheet of material around its longitudinal axis. While the process of formation of the octopus spermatophore has not been observed, the structure of the spermatophoric organ indicates that, as in the squid, the forming spermatophore is kept rotating on its longitudinal axis while a thin sheet of secretion is supplied and wound on, like the successive layers of fabric in a rubber hose. During ejaculation the middle membrane is shown to be very pliable, evidently much more so than in the squid.

Orally, the middle membrane is firmly united to the outer tunic, where this tunic is joined by the cap (fig. 2 MM′). Toward the aboral extremity of the ejaculatory apparatus the middle tunic becomes thinner and almost if not quite disappears before the extremity is reached.

Inside the middle membrane is the inner membrane. This is so thin that in all the figures it has been shown as a single line, but it is always distinguishable under a moderately high-power lens. This is also firmly united to the outer tunic at its oral extremity (fig. 2 IM) and extends throughout the length of the ejaculatory apparatus.

Inside the inner membrane is a spirally coiled filament that is present but hard to distinguish near the oral end of the ejaculatory apparatus and becomes very much more prominent toward the aboral end of the ejaculatory apparatus (fig. 1 SF). This filament seems to be united to the inner membrane and has the appearance of an ornament on the inner membrane. It is evidently very flexible and does not break up into small fragments during ejaculation, as in the squid spermatophore. While it possesses elasticity and probably aids in keeping the ejaculatory apparatus from collapsing, there is no evidence that it possesses any spring properties. The diameter of the coil differs greatly. Toward the aboral end of the ejaculatory apparatus, where the middle membrane thins and probably disappears, the diameter of the coil of the spiral filament is greatly increased. It then narrows and near its aboral end becomes thin and relatively weak. This arrangement is significant in the act of ejaculation.

The lumen of the ejaculatory apparatus inside the spiral filament is filled with a viscid material that adheres to the spiral filament and inner membrane and becomes spread over the outside in an irregular manner during ejaculation (figs. 2, 12, and 15 HC). It is not so liquid as in the squid spermatophore and does not form a definite rod-like plug like that described by Racovitza (1894 c) for *Rossia*. There is frequently evidence that the core of this secretion is much more liquid than the outer parts (fig. 2 HC′). Extending into the cap of the spermatophore from this region is an indefinite, hazy appearance evidently due to the escape of material from the lumen. This appearance never involves the entire width of the lumen but only the central part. The remainder of the material is evidently responsible for forming the papillæ-like ornaments over the outside of the evaginating tube that,

as evagination continues, becomes a more or less definite layer, often with lump-like masses that cover the outside (figs. 2, 7, 12, and 15).

About one-third of that portion of the ejaculatory apparatus nearest the oral end of the spermatophore is spirally coiled. There are from 14 to 20 distinct loops in this coil. The loops are pressed close together, but there is a distinct groove between them that is filled with the granular liquid. There is considerable difference in spermatophores as to how near the oral end the coiling begins. Frequently (as shown in figure 1) there are a number of loose coils near the oral end, but this is not always the case. When ejaculation begins the loops near the oral end are first to straighten out.

EJACULATION.

When a spermatophore is placed in sea-water and the cap thread is pulled, ejaculation begins immediately. Ejaculation will begin in spermatophores placed in sea-water without pulling the thread, but the process is delayed. This is doubtless due to largely increased tension due to osmosis and may be aided by softening of the cap. Ejaculation will also begin in the air, probably because drying shrinks the outer tunic and thus increases the tension, but this is of course not normal.

It is easiest to keep material for study in a solution of calcium chloride and to remove specimens individually into sea-water when needed for study. Specimens will keep in good condition for study in this solution for some hours. Many other solutions that reduce the osmotic tension in the spermatophores have bad effects on the membranes so that normal ejaculation is interfered with when they are returned to sea-water.

It is probable that normal ejaculation is started by pulling the cap thread. How this is done has not been ascertained. The spermatophores are stored in the spermatophoric sac with their aboral ends pointing outward—that is, toward the opening of the penis. *Octopus* has a hectocotylized arm that has a groove passing along one margin from the base to the tip. There is every reason to believe that the contents of the spermatophores, if not the spermatophores themselves, are passed along this groove from the base to the tip.

Racovitza (1894 *a* and *b*) has described how in *Octopus vulgaris* the tip of this arm is inserted into the mantle cavity of the female, and he found by dissection afterward that the oviducts of the female were packed with sperm. In 95 minutes during which the act of copulation continued no movements of the animals were observed except slight movements of the hectocotylized arm. Although the actual passage of the sperm could not be seen, as the arms are very opaque, it is evident that they are passed down this groove.

Racovitza speaks of finding the "spermatophores" in the oviducts, but later he modifies this by stating that the examination of the spermatophores showed their sheaths had disappeared and that only the part evaginated persisted. He states further that they had been placed by the orifices of the oviducts and in exploding introduced the spermatic reservoirs into the canal.

The statement concerning the condition of the sperm in the oviducts corresponds with my observations made on a specimen at Montego Bay, in which the oviducts were filled except that there were no reservoirs. The sperm were free. There were no parts of the tunics or ejaculatory apparatus found. I do not know upon what evidence Racovitza concludes that the spermatophores were placed by the orifices of the oviducts and in exploding introduced the spermatic reservoirs into the canal. Very possibly it was surmise based on known conditions in other forms. There is no known provision for sticking the spermatophores to the body of the female and there is no spermatic reservoir formed in ejaculation in *Octopus*. It seems probable either that the ejaculating spermatophore is held by the tip of the hectocotylized arm in position for it to introduce the sperm mass into an oviduct as it ejaculates, or that the spermatophore never enters the groove in the hectocotylized arm, but ejaculates into it, and the sperm mass *only* passes on to the tip and thus into the oviduct. In either case the spermatophores evidently have to reverse ends in passing from the penis to the groove in the hectocotylized arm, for the oral ejaculating end must be directed toward the oviduct while it is functioning. What mechanism is used in transferring the spermatophore from the penis to the groove is not known, but it is probable that during the process the thread is pulled and ejaculation begins.

Ejaculation is very deliberate, occupying from $1\frac{1}{2}$ to 3 minutes, and the sperm thread is all unwound, so that it leaves the spermatophore as a long narrow thread. Neither of these arrangements seems to be adapted to packing the oviducts directly from the ejaculating spermatophores, but they are nicely adjusted to delivering the sperm thread to the groove in the hectocotylized arm. I therefore doubt somewhat whether the spermatophore ever enters the groove. It seems more probable that it is retained between the arms near the entrance to the groove and that the sperm mass only, in the form of the uncoiled thread, passes down the groove to the tip of the hectocotylized arm. The tip of this arm seems to be modified for introduction into the oviducts and by it the sperm would be conducted into position.

The cap thread, as stated in describing the structure of the spermatophore, forms a broad bandage, one end of which is quite firmly stuck to one side of the spermatophore, while the other end is free but passes over the end of the cap. The free end of the thread is

among the other spermatophores in the spermatophoric sac, and as they move aboral end first toward the penis these threads are in position to be pulled when the spermatophores leave the penis.

Arrangement near the base of the penis indicates that a single spermatophore is ejected at a time.

When the cap thread is pulled the cap immediately begins to swell and elongate (fig. 7), and the evagination of the ejaculatory apparatus begins. The cap soon goes to pieces (fig. 8) and evagination continues. The middle membrane is very pliable and is extended far beyond the point of folding back (figs. 7, 10, 11, and 14 PT).

The hyaline core is composed of two parts, a central core (HC′) that is quite liquid mixes with the water and disappears, and an outer part (HC) that sticks to the inner membrane forming small projections and lumps (figs. 7, 12, and 15) that change form considerably as ejaculation is continued.

At the beginning of evagination the spiral coiling of the ejaculatory apparatus begins to straighten out. The loops nearest the cap are affected first, and little change is noticeable in the positions of other parts in the spermatophore until all these coils have been straightened (figs. 11 and 12). The twist in the ejaculatory apparatus is shown again in evagination by the spiral into which the tube is again thrown at the evaginating extremity (figs. 11, 12, and 13). When a position about equivalent to the point 4, figure 1, is reached the act of evagination is slowed down considerably. This is probably due to resistance offered by the spiral filament which here becomes better developed. The slowing and the torsion developed by evaginating the spiral filament result in swelling the evaginating end of the tube, crowding and spirally coiling the aboral end of the ejaculatory apparatus into the swelled portion, and in uncoiling the sperm thread and pressing it forward into the part of the tube that has evaginated (fig. 16).

The resistance to evagination becomes greater in the region where the spiral filament is best developed and the swelling of the evaginating end is correspondingly increased. This continues until the sperm thread reaches the portion of the ejaculatory apparatus that is still to evaginate and becomes crowded along its sides.

When evagination reaches the point where the middle membrane thins and disappears it becomes very much more rapid, the remainder is turned rapidly, and the sperm thread immediately begins to escape through the open evaginated end of the ejaculatory apparatus. It commonly happens (fig. 17) that the ejaculatory apparatus ruptures near its narrowed extremity and that the escape of the sperm thread takes place through this rupture.

The escape of the sperm thread is even, not hurried, and continues for a minute or more. It begins while a considerable portion of the aboral end is still closely coiled. The coils open one after another and

the thread moves continuously on until it is entirely free from the outer case. The thread as it uncoils must have a somewhat similar twisting and this probably accounts for the alternate swellings and constrictions along the thread. This appearance is very much more marked near the coils than toward the free extremity where the twist would have time to adjust itself somewhat. Focussing on the narrow parts shows the places of twisting marked by wrinkles and striations in the mass.

The outer tunic does not shrink nearly as much as it does in the squid spermatophore and the middle tunic does not swell nearly as much. Indeed they hardly seem to give evidence of all of the power that is needed for ejaculation. The whole process is very deliberate. It is very possible that the liquid that occupies the space between the tunics and the ejaculatory apparatus and sperm mass has osmotic properties that are important in the process of ejaculation.

In the spermatophore before ejaculation much lumpy, granular material is present in this liquid. This seems to become much more liquid as ejaculation continues. It would seem that most of this would be thrown out ahead of the sperm thread, but this is not the case. The end of the sperm thread is crowded in against the ejaculatory apparatus in such a way that, when this finally evaginates and leaves a free opening to the outside, the sperm thread fills this opening at once and the liquid remains inclosed between it and the evaginated tube. It is not free to escape in quantities until the sperm mass has unwound and the thread of which it was composed has been entirely discharged.

Evidently the spermatophores of *Octopus* and the squid, while built on similar plans, are adapted for quite different actions. It may be of service to call attention to some of the important differences.

The squid spermatophore is adapted for very quick service and for filling a reservoir with the entire mass of sperm and sticking it to the body of the female. The contents slowly escape from the reservoir and are stored in a special receptacle, or fertilize the eggs as they leave the oviduct.

The octopus spermatophore is adapted for very deliberate service. The sperm mass is not stored in a special reservoir and there is no provision for sticking it to the body of the female. The sperm are introduced directly into the oviducts of the female, where they are stored until the eggs are ready to be laid.

The squid spermatophore is accordingly more complicated in structure and is under greater tension than that of *Octopus*. It has a cement body, which is entirely absent in *Octopus*. It has an outer membrane and inner tunic that are used in making a sperm reservoir, and has these structures attached to the cement body, so it may be ruptured at the right time. These are absent in *Octopus*. The sperm thread is also closely packed and bound into a mass that is inclosed in

the aboral portion of the inner tunic, and the whole mass is moved together. The sperm thread of which it is composed is never uncoiled. This differs entirely in *Octopus*, where the sperm thread is not closely packed, is not inclosed in any kind of membrane, and uncoils as it is discharged.

These questions of structure and speed are of course related to the functions they perform. Squid are free-swimming, active creatures, and copulation is a very rapid act. *Octopus* are for the most part bottom dwellers and copulation is much more deliberate. The quick machine is more complicated, but the two show very similar structure.

Without knowledge of the past history of these animals it is very difficult to arrive at reasons for the formation of such complicated structures as these spermatophores to perform functions where simpler arrangements would seem to do as well. Doubtless if the past history were before us for review we would see clearly why such structures have been developed, but in the absence of this history which seems to be permanently hidden from us, we can be certain only that the complicated machine has been formed and serves its purpose.

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The papers given are the only ones to which it has been necessary to refer in this paper. There are many others.

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EXPLANATION OF FIGURES.

The figures were all drawn with the aid of a camera lucida from specimens preserved in formalin and for the most part stained and then mounted in glycerine jelly. Stages necessary for study and drawing were secured by deluging with full strength formaldehyde at the moment required. Large spermatophores before ejaculation may measure 50 mm. in length. The process of ejaculation increases the length to over 100 mm. and to this should be added the sperm thread, which, when uncoiled and ejected, measures at least 150 mm. more.

ABBREVIATIONS.

C, cap.
CT, cap-thread.
HC, hyaline core; the outer stiff portion of the core only is referred to.
HC′, hyaline core; the inner, more liquid material that mixes with the water and disappears is referred to.
MM, middle membrane
MM′, middle membrane; the point of attachment to the outer tunic.
MT, middle tunic; this extends orally only a little further than the sperm mass.
OT, outer tunic.
PT, point of turning of the middle membrane; during ejaculation the pliability of membranes is such that this point lags far behind the outermost tip of evagination.
SF, spiral filament.
SL, space filled with liquid; this space is inclosed by the outer and middle tunics on the outside and extends between these structures and the sperm mass and ejaculatory apparatus; as ejaculation takes place the space continues down the evaginating ejaculatory apparatus.
ZM, sperm mass; this consists of a spirally coiled sperm-thread that uncoils during ejaculation.
S, granular material in the space, SL.

PLATE I.

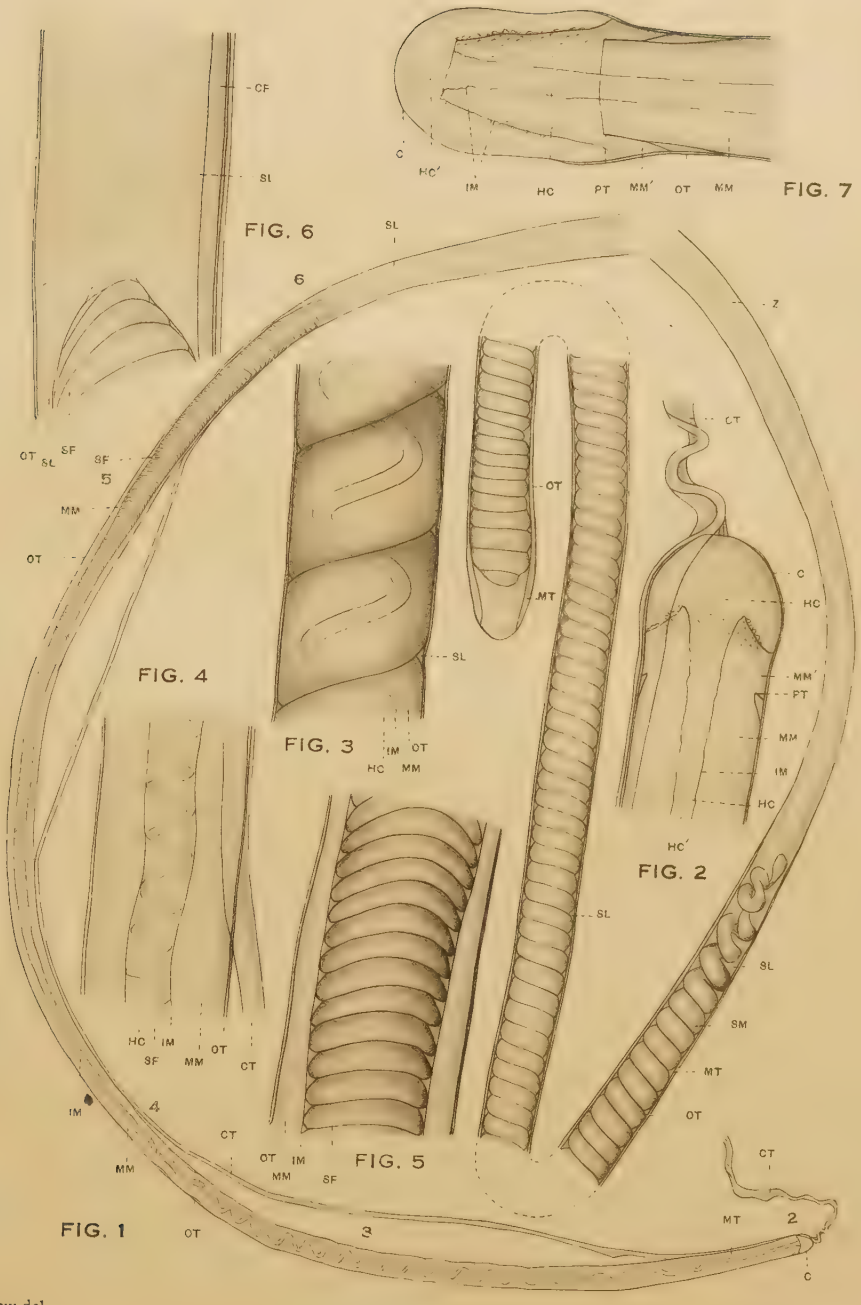
1. Spermatophore as taken from the spermatophoric sac, × 10 diameters. The specimen has been drawn in sections and the connections indicated by dotted lines. Specimens just removed from the spermatophoric sacs frequently show the sperm mass entirely filling the aboral end to the tunic. It is common to have specimens in which the ejaculatory apparatus, which extends from point 2 to point 6, is closely coiled nearer the oral opening than was the case in this specimen. The numbers 2, 3, 4, 5, and 6 placed along the side of this drawing indicate the positions of more highly magnified drawings that bear the same numbers.
2. Oral end of the specimen shown by figure 1, × 63 diameters. The position is indicated by the side of figure 1 by the number 2.
3. A portion of the specimens shown by figure 1, × 63 diameters. The position is indicated by the side of figure 1 by the number 3.
4. A portion of the specimen shown by figure 1, × 63 diameters. The position is indicated by the side of figure 1 by the number 4.
5. A portion of the specimen shown by figure 1, × 63 diameters. The position is indicated by the side of figure 1 by the number 5.
6. A portion of the specimen shown by figure 1, × 63 diameters. The position is indicated by the side of figure 1 by the number 6.
7. The oral extremity of a spermatophore at the beginning of ejaculation, × 63 diameters. The cap-thread has been stripped back so that it does not show in this figure. This frequently happens in handling the specimens before they are permanently mounted. Soon after this stage is reached, the cap bursts and the fragments disappear. The cone-like projections on the portion extending into the cap are formed by the outer portions of the hyaline core which becomes spread over the outer surface of the evaginating tube. They settle down and are not as prominent after ejaculation has proceeded some distance.

PLATE II.

8. Oral end of a spermatophore after the cap has broken and disappeared, × 63 diameters. A ridge, which is probably more properly referred to as the margin of the outer tunic, remains behind and for convenience in marking the position is labeled in this and subsequent figures the same as the cap by the letter C. The liquid material of the hyaline core HC′ is shown being liberated into the water.
9. Oral end of a spermatophore at a slightly more advanced stage in ejaculation, × 14 diameters.
10. Oral end of the spermatophore shown by figure 9, × 63 diameters. It frequently happens, as shown in this figure, that the ridge to which the cap was attached (C) is reflected so that the free edge points backward. This is evidently due to the force exerted on the outer tunic by the attached middle membrane which draws the tunic in somewhat and by the enlargement of the softer membranes immediately upon leaving the confining outer tunic. The wrinkling that is shown near the point of turning of the middle membrane (PT) is pronounced as soon as the turning is well started. It is evidently due to the fact that the middle membrane is made up of a thin sheet wound around the central hyaline core. This allows some coats of the tunic to wrinkle while others are stretched. In this as in other specimens the reflecting of the individual layers can be traced from the point PT to the free evaginating end. They have not been shown in the figures, as they would be confusing.
11. Oral end of a spermatophore at a slightly more advanced stage of ejaculation than the one shown by figure 10, × 24 diameters. As the ejaculatory apparatus uncoils the torsion caused by the evaginating tube throws its extremity into a spiral that becomes longer and more pronounced the more the original coil is straightened.
12. Oral portion of a spermatophore during ejaculation, × 12 diameters. The stage is more advanced than that shown by figure 11. The original spiral coiling of the ejaculatory apparatus has straightened and the torsion that causes the end of the evaginating tube to be thrown in a spiral is accordingly at its greatest. This specimen shows the lumps of material derived from the hyaline core adhering to the evaginated tube more prominently than is usually the case. The numbers 13, 14, and 15, placed along the side of this drawing, indicate the positions of more highly magnified drawings that bear the same numbers.
13. Extremity of the specimen shown by figure 12, × 50 diameters. The position is indicated by the side of figure 12 by the number 13.
14. Region of the point of turning of the specimen shown by figure 12, × 50 diameters. The position is indicated by the side of figure 12 by the number 14.
15. A portion of the evaginating ejaculatory apparatus of the specimen shown by figure 12, × 50 diameters. The position is indicated by the side of figure 12 by the number 15. This shows a large mass of the material derived from the hyaline core adhering to the surface.

PLATE III.

16. A spermatophore that has evaginated to the point where the ejaculatory apparatus retards the evagination until the pressure swells the end that is evaginating, × 10 diameters. The specimen has been drawn in sections and the connections indicated by dotted lines. The remaining portion of the ejaculatory apparatus has been thrown into a spiral coil, probably because of the torsion exerted by evagination together with the pressure from behind. The sperm thread that forms the sperm mass has uncoiled for some distance. At a little later stage a considerable portion of the remaining ejaculatory apparatus is evaginated and the sperm thread is pushed down against it. At this time the swelling at the end is much greater than that shown in this figure. When the evagination of the ejaculatory apparatus is complete the sperm thread is set at liberty and immediately begins to push out through the opening.
17. Extremity of the fully evaginated ejaculatory apparatus with the sperm thread escaping, × 24 diameters. In this specimen, as frequently happens, the side of the ejaculatory apparatus has ruptured near its extremity and the sperm thread is escaping through the rupture. Some of the granular liquid is seen escaping by the side of the sperm thread.


CF
SL
FIG. 6
C
HC'
IM
HC
PT
MM'
OT
MM
FIG. 7
SL
6
Z
OT
SL
SF
SF
5
MM
OT
FIG. 4
OT
MT
SL
FIG. 3
IM
OT
HC
MM
CT
C
HC
MM'
PT
MM
IM
HC
HC'
FIG. 2
SL
SL
SM
MT
OT
HC
IM
SF
MM
OT
CT
4
IM
CT
OT
IM
MM
MM
SF
FIG. 5
CT
MT
2
FIG. 1
OT
3
C

Drew del.

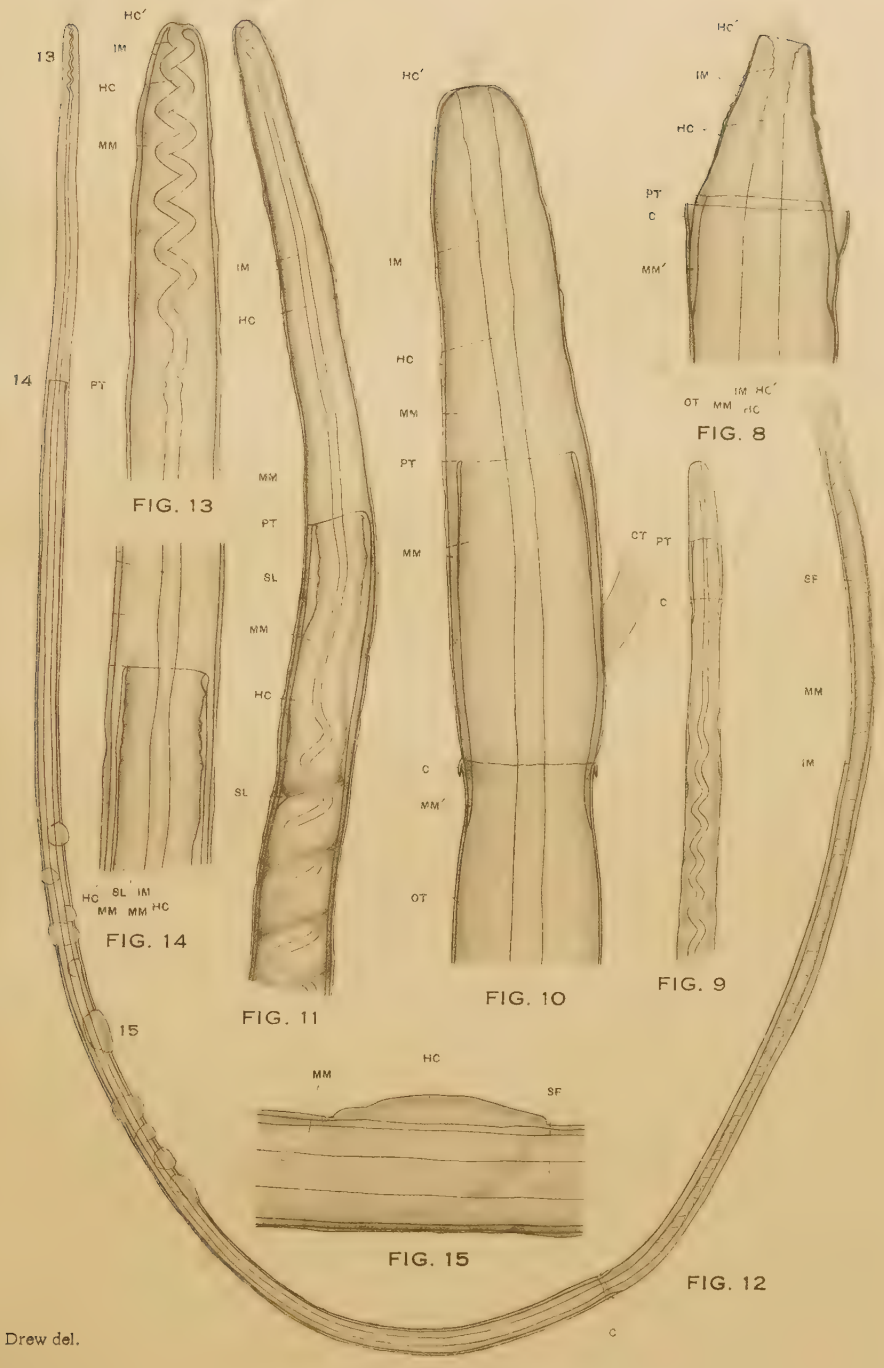


FIG. 13

FIG. 14

FIG. 11

FIG. 10

FIG. 8

FIG. 9

FIG. 15

FIG. 12

Drew del.

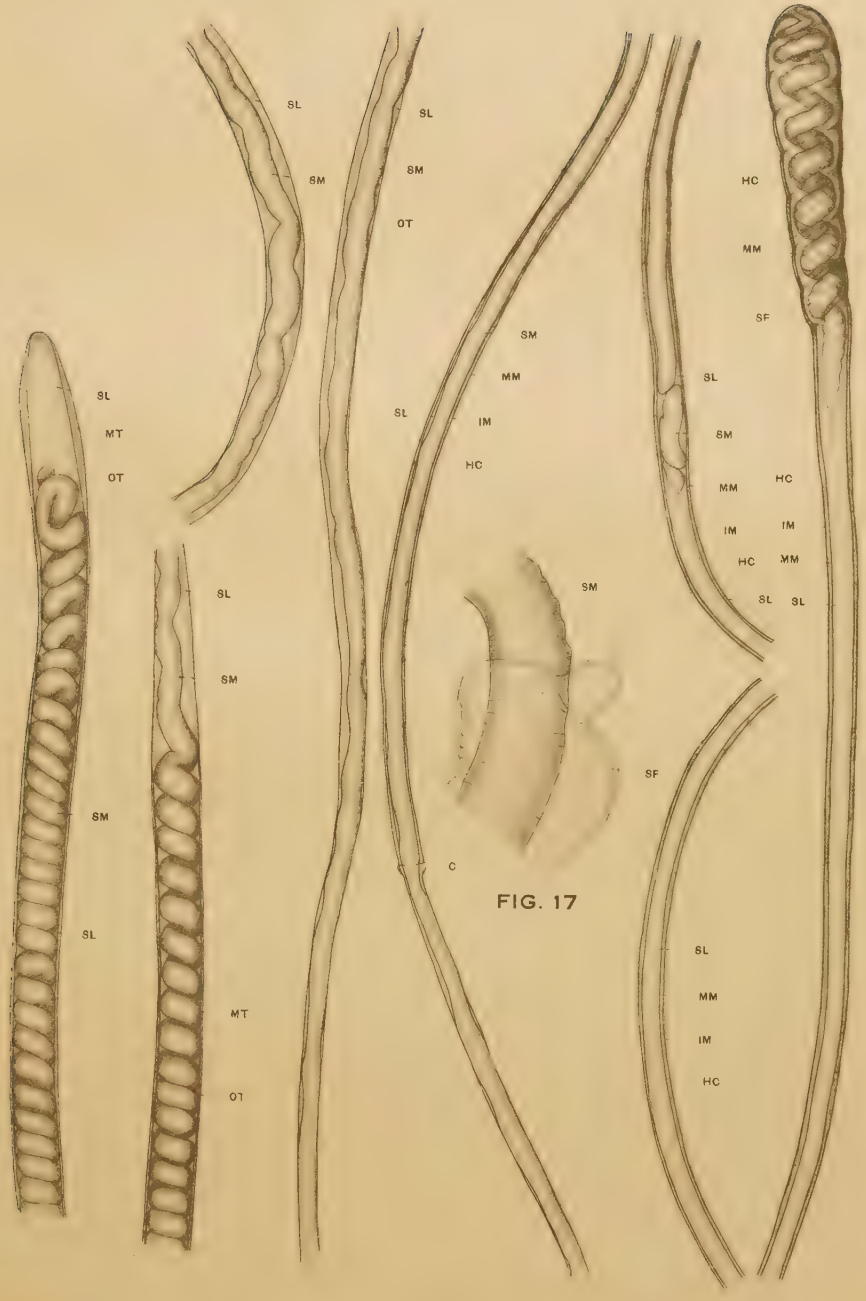


FIG. 17

FIG. 16

Drew del.

III.

THE DISTRIBUTION OF THE LITTORAL ECHINODERMS OF THE WEST INDIES.

By HUBERT LYMAN CLARK,

Of the Museum of Comparative Zoölogy, Cambridge, Massachusetts.

Three plates.

THE DISTRIBUTION OF THE LITTORAL ECHINODERMS OF THE WEST INDIES.

By Hubert Lyman Clark.

INTRODUCTION.

Among the various groups of invertebrate animals which swarm on the reefs and along the shores of the West Indies none is more conspicuous than the Echinodermata and few are more abundant or diversified. Wherever conditions are at all favorable for the formation of coral reefs, and in many places where corals scarcely grow at all, echinoderms are found in greater or less abundance, and either by their size or color or both are sure to attract the attention of even a casual observer.

In April 1896 I first came into contact with this interesting fauna while I was enjoying the privileges of the Johns Hopkins University's Marine Laboratory at Port Henderson, Jamaica. The following summer my acquaintance with it was renewed at Port Antonio, Jamaica, under the same auspices.

In April 1899 I spent two weeks in Bermuda, most of my collecting being in the vicinity of Bailey Bay, Coney Island, and Castle Harbor. In the fall of 1902 I again visited Port Henderson, Jamaica, and in the spring of 1909 I was at Port Antonio again for a week. In the spring of 1912 I enjoyed the privileges of the Carnegie Institution's laboratory at Montego Bay, Jamaica, while in 1916, under the same auspices, I had the opportunity of spending five weeks at Pigeon Point, Tobago. Finally, the month of June 1917 was spent at the Tortugas Laboratory of the Carnegie Institution of Washington, where every facility was provided for the collection and study of echinoderms.

As a result of these unusual opportunities, I have accumulated a large part of the data presented in this report, my attention having been almost wholly given to the littoral echinoderms. The word *littoral* is used in the strictest sense, only those species being included which I have myself collected on the reefs or in very shallow water, or for whose occurence there the records are indubitable. As a matter of fact, I have collected nine-tenths of these species. In compiling the list, I have been very arbitrary and have omitted a considerable number of species which might be expected in it. Further reference will be made to these under the respective groups into which the list is divided. The collections of the Museum of Comparative Zoölogy contain a large amount of West Indian material and this has been freely used and of invaluable assistance in the preparation of this paper. I have also made use of all available publications in search-

ing for records for the different islands, but no doubt scattered records have been overlooked, while many in which I have lacked confidence are ignored.

The purpose of this investigation and report is to see if the distribution of these very littoral species throws any light on the faunistic relations of the various islands. But it should be understood at the start that we have nowhere nearly enough data on which to base any important conclusions. Thus, while the littoral echinoderms of Florida and the Tortugas are quite completely known and those of Jamaica are almost equally well listed, our knowledge of the Cuban fauna is, in this group, very incomplete and nothing whatever is known of the Isle of Pines or of the Cayman Islands. Of the echinoderms occurring on the shores of Porto Rico and the United States Virgin Islands, we are fairly well cognizant, but passing eastward and southward we enter a region of which our knowledge is most superficial until Barbados and Tobago are reached. This report is therefore merely introductory to the subject, but it is hoped that it may serve as a useful beginning.

One word is necessary as to the geographical limits of the region here designated as "West Indies." It extends from Bermuda on the north and the Tortugas on the west to Tobago in the southeast. Perhaps, strictly speaking, these three extremes do not belong in the region at all, but as their littoral faunas are exceptionally well known it would be absurd to leave them out of account.

In concluding this introduction, I wish to express my thanks to those whose encouragement and aid have made my work possible. I desire particularly to record my great and lasting obligations to the late Professor William Keith Brooks, of the Johns Hopkins University, who first opened to me the fascinating field of marine zoölogy; to the late Dr. Alexander Agassiz and to Mr. Samuel Henshaw, of the Museum of Comparative Zoölogy; and to Dr. Alfred G. Mayor of the Carnegie Institution. At Tobago I had the privilege of the constant companionship and help of my honored colleague, Dr. Th. Mortensen, of Copenhagen; it is a pleasure to acknowledge here my debt to him. Another colleague, Dr. W. K. Fisher, of Leland Stanford Junior University, has put me under obligation by permitting me the use of certain field notes made by him during the summer of 1918 at Barbados and Antigua, where he was a member of the party sent out by the University of Iowa, under Professor C. C. Nutting. I am glad to thank Professor Nutting and Dr. Fisher for permitting me to use these notes.

There are many to whom my thanks are due for help in collecting, but I forbear attempting to name them. I must, however, offer my particular thanks to Mr. John W. Mills, chief engineer of the Tortugas laboratory, whose interest and help have been invaluable.

I. WEST INDIAN LITTORAL ECHINODERMS.

Recent echinoderms fall so readily into their five classes, without any disconcerting annectant forms, that it is easiest and most natural to discuss each class separately.

COMATULIDA. FEATHER-STARS.

At Tobago we found a comatulid, *Tropiometra carinata* (Lamarck), common in very shallow water in Buccoo Bay and on Buccoo Reef. It is a species of wide distribution on the coasts of southern Africa and Brazil, and reaches its northern limit in deep water (200 to 300 fathoms) off St. Lucia. As a littoral species, its northern limit seems to be at Tobago. The only other littoral comatulids of the West Indies are species of the genus *Nemaster*, though Mr. A. H. Clark tells me that the little-known *Antedon dübenii* Böhlsche, of Brazil and St. Thomas, appears "to be from very shallow water." The remarkable and unique occurrence of *Nemaster iowensis* (Springer) at the Tortugas "in water less than 3 feet deep" in 1893, is one of the most curious facts of distribution which the West Indian region affords. Its mystery is only deepened by the apparent occurrence of the same species at Bermuda. Mr. A. H. Clark very kindly permits me to thus note the fact that he has a specimen from that northern point. It is certain that other species of *Nemaster* occur in the West Indies, as is indicated by material in the United States National Museum, but as yet our knowledge of the genus is too fragmentary to make it of any use in discussing distribution. So *Tropiometra* is the only comatulid at present of value for this purpose and its occurrence at Tobago distinguishes that island at once from the other West Indies.

ASTEROIDEA. SEA-STARS.

The littoral sea-stars of the West Indies are not numerous in species or abundant as individuals, and it is only occasionally or under special conditions that a species can be called common. Under favorable conditions, in particular spots, *Astropecten duplicatus* is very abundant, but these spots are as a rule in water more than 3 fathoms deep. On the other hand, *Asterina folium* occurs at or just above low-water mark, on particular reefs, in sufficient numbers to warrant calling it common, and the same is sometimes true of young individuals of *Linckia guildingii*, and at Bermuda it is true of *Coscinasterias tenuispina*. At Port Royal, Jamaica, *Echinaster sentus* is fairly common and *Oreaster reticulatus* is by no means rare. Verrill reports the latter species as "very common in the Bahamas." No other sea-stars can be called common in the shallow waters of the West Indies, but *Ophidiaster guildingii* is widely distributed and is by no means rare on the Tortugas reef-flats and on Buccoo Reef, Tobago.

The following 14 species make up the list of littoral sea-stars of the West Indies. All have been taken at or near low-water mark, certainly in less than 3 feet of water.

Astropecten articulatus (Say).	Asterina minuta Gray.
duplicatus Gray.	Stegnaster wesseli (Perrier).
Luidia alternata (Say).	Ophidiaster guildingii Gray.
clathrata (Say).	Linckia guildingii Gray.
senegalensis (Lamarck).	Echinaster sentus (Say).
Oreaster reticulatus (L.).	spinulosus Verrill.
Asterina folium (Lütken).	Coscinasterias tenuispina (Lamarck).

The West Indian species of *Astropecten* are exceedingly perplexing, although Verrill has recently (1915, Bull. Univ. Iowa, Lab. Nat. Hist. 7, pp. 152–187) made an important contribution to their elucidation. Lütken's *A. antillensis* has been often recorded, but Verrill thinks it is possible that this nominal species is identical with *duplicatus*. If he is correct, it is obvious that the forms I listed from Porto Rico as *antillensis* (1901, U. S. Fish Com. Bull., 2, p. 236) are certainly not that species. They are perhaps Verrill's *A. comptus*. All the astropectens which I have myself collected at the Tortugas, in Jamaican waters, and at Tobago are referable to either *articulatus* or *duplicatus*, and I am inclined to consider these the only two strictly littoral species.

Conditions in the genus *Echinaster* are equally bad. A number of species are listed from Florida and from Brazil, and several of these are recorded from Cuba, Jamaica, Haiti, Porto Rico, and St. Thomas. My experience in Jamaica has satisfied me that there is only one littoral species there and, so far as I can see, it is not distinguishable from the common Florida species, *sentus*. I have previously called it *spinosus*, but Verrill has pointed out that no species of *Echinaster* may properly bear that name. The material in the Museum of Comparative Zoölogy shows that *sentus* is a very variable species and it may be that a more satisfactory knowledge of it will permit the recognition of varieties and possibly of subspecies. So far as I can see, no other species of *Echinaster* is known from the West Indies proper, but on the west coast of Florida, as far south as the Tortugas, there is a second well-marked species, *spinulosus* Verrill. There are specimens, undoubtedly *spinulosus*, in the Museum of Comparative Zoölogy labeled as coming from off Cape Fear, North Carolina, in 7 fathoms, but there is a possibility of a mistaken label, and the record may be ignored until confirmed by the discovery of additional specimens north of Florida. Verrill says he has seen no specimens from the eastern coast of Florida, and of those in the Museum of Comparative Zoölogy, all (save the lot mentioned) are from western Florida or Louisiana.

There can be little doubt of the occurrence of two species of *Asterina* in the West Indies, as I have found both in Jamaica and at Tobago,

and Hartmeyer found both at Barbados. Verrill doubts the distinctness of the two forms and calls both *folium*. Döderlein (1910, Zool. Jahrb. Supp. 11, pp. 152–155) gives the name *minuta* to the form with 2 to 4 spinelets on the actinolateral plates, and describes the form with a single such spinelet as a new species, *hartmeyeri*. As has long been known, both forms were regarded by Gray as varieties of Linné's *Asterias minuta*, but in 1859 Lutken gave the name *folium* to the form with 2 to 4 actinolateral spinelets, leaving the name *minuta* for the other. Hence Döderlein's proposed name seems to me quite superfluous.

Verrill (1900, Trans. Conn. Acad., **10**, p. 584) gives *Ophidiaster guildingii* as occurring at Bermuda, but this is obviously a slip of the pen, *Linckia guildingii* being the species he had in mind. The little sea-star taken by me at Port Antonio, Jamaica, in 1897 and listed (1898, J. H. U. Circ., No. 137, p. 5) as *Pentagonaster parvus* is of very uncertain identity; it will probably prove to be a young *Oreaster reticulatus*, the growth stages of which are at present almost wholly unknown.

Of the 14 sea-stars listed above, one appears to be tropicopolitan and hence of little service in determining faunal areas within the tropics; this is *Linckia guildingii*. I have examined specimens from the Society Islands; Masthead Island, Queensland; Zanzibar; and Lower Guinea, as well as much material from the West Indian region, and I have not discovered any valid specific differences.

Of the remaining 13 species, *Luidia alternata*, *L. clathrata*, and *Oreaster reticulatus* are widely distributed in the tropical Atlantic from South Carolina to Brazil, and *Oreaster* occurs even in the eastern Atlantic. A similar but somewhat more southern range is that of *Luidia senegalensis*, which, although known from the west coast of Africa, does not occur at Bermuda or on the coast of the United States north of Florida.

Of the remaining species, the following 5 are distinctly characteristic of the West Indian region: *Astropecten duplicatus*, *Asterina folium*, *A. minuta*, *Stegnaster wesseli*, and *Ophidiaster guildingii;* while *Astropecten articulatus*, *Echinaster sentus*, and *E. spinulosus* seem to be restricted to the northern part of the region. If, however, it is true, as reported, that *A. articulatus* really occurs at Dominica and Martinique, and *E. sentus* actually lives on the coasts of Brazil, which seems improbable, this distinction has no validity, for *E. spinulosus* is a very local species, probably confined to the northeastern coasts of the Gulf of Mexico.

The fourteenth species, *Coscinasterias tenuispina*, is distinctly a Mediterranean and eastern Atlantic form. It is common at Bermuda, but it is not impossible that it was introduced there accidentally.

Verrill reports it from Cuba and from Brazil and even says "West Indies." He also describes a variety (originally a species) *atlantica*, the type of which is said to be from Cuba. The occurrence of a littoral *Coscinasterias* in Cuba, however, needs to be confirmed, for it is many years since Verrill's specimens were taken and nothing of the kind has been found in that region since.

OPHIUROIDEA. BRITTLE-STARS.

The littoral brittle-stars of the West Indies are numerous in species and very abundant as individuals. Both *Ophiactis savignyi* and *Ophiothrix angulata* swarm wherever conditions are suitable, while every reef which supports echinoderm life at all is sure to abound with ophiocomas and ophiodermas. On a reef where conditions are moderately favorable, one may be reasonably sure of finding, anywhere in the West Indies, at least 15 species of brittle-star and, on an exceptionally good ground, such as Buccoo Reef, Tobago, the number may rise to 25.

It is not always easy to decide whether a given species of brittle-star should be included in the present list or not, but where a species is well defined and well known from a few fathoms depth, like *Ophiothrix lineata*, I have included it, even though I have not found it in water less than a fathom deep. On the other hand, where the specific limits of a species are ill-defined and hence the reliability of the records are dubious, as with *Ophiactis mülleri* Lütken, I have omitted it unless I have myself collected it along the shore or on a reef. I have omitted also *Ophiopsila hartmeyeri* and *Ophiolimna littoralis*, recorded by Kœhler from St. Thomas, and *Amphiodia gyraspis*, recorded by me from Porto Rico, because they are not littoral in the narrow sense used in this paper. Several other Porto Rican species whose identification is dubious are likewise ignored.

The following 58 species are those to which the title "littoral brittle-stars of the West Indies," in my judgment, rightfully belongs:

Ophiomyxa flaccida (Say).
Astrophyton muricatum (Lamarck).
Ophiacantha oligacantha H. L. Clark.
Ophiomitrella glabra (H. L. Clark).
Amphiura kukenthali Kœhler.
palmeri Lyman.
stimpsonii Lütken.
vivipara H. L. Clark.
Hemipholis elongata (Say).
Ophiophragmus filograneus (Lyman).
lütkeni (Ljungman).
pulcher H. L. Clark.
septus (Lütken).
wurdemanii (Lyman).
Ophionephthys limicola Lütken.
Ophionema intricata Lütken.
Amphipholis gracillima (Stimpson).
pachybactra H. L. Clark.
squamata (Delle Chiaje).
Ophiostigma isacanthum (Say).
Amphiodia planispina (von Martens).
pulchella (Lyman).
repens (Lyman).
rhabdota H. L. Clark.
trychna H. L. Clark.
tymbara H. L. Clark.
Ophiocnida scrabriuscula (Lütken).
Amphioplus abditus (Verrill).
coniortodes H. L. Clark.
thrombodes H. L. Clark.

Ophiactis cyanostictà H. L. Clark.
lymani Ljungman.
savignyi (Müller & Troschel).
Ophiothrix angulata (Say).
brachyactis H. L. Clark.
lineata Lyman.
œrstedii Lütken.
suensonii Lütken.
Ophionereis olivacea H. L. Clark.
reticulata (Say).
squamulosa Kœhler.
Ophiocoma echinata (Lamarck).
pumila Lütken.
riisei Lütken.
Ophiopsila riisei Lütken.
vittata H. L. Clark.
Ophioderma appressum (Say).
brevicaudum Lütken.
brevispinum (Say).
cinereum Müller & Troschel.
guttatum Lütken.
januarii Lütken.
phœnium H. L. Clark.
rubicundum Lütken.
squamosissimum Lütken.
Ophiozona impressa Lütken.
Ophiolepis elegans Lütken.
paucispina (Say).

While certain of the above species are subject to great diversity, especially in color, it is fortunately true that nearly all, even in such genera as *Amphiura* and *Ophiothrix*, are recognizable with comparatively little difficulty. Specific limits in the genus *Ophiactis* are not very well defined; in particular, the species *mülleri* and *savignyi* have been much confused and young individuals are certainly hard to separate. Adult *mülleri* seems, however, to be a much larger and darker-colored animal than adult *savignyi* and, so far as I can learn, it inhabits deeper water and does not occur on the reefs accessible at low tide. It is therefore ignored in this list. The genus *Ophiothrix* contains the most ill-digested assemblage of species of any genus of ophiurans, but the littoral West Indian species are not hard to separate when once their distinctive characters are understood. The extraordinary diversity of color and disk spinulation shown by *O. angulata* is certainly perplexing but each of the other species has distinguishing characters which are quite easy to see at once. In the genus *Ophioderma*, the species *appressum* and *brevispinum* are so similar that they are often confused. Typical examples are not difficult to distinguish, but each form is variable and some of the varieties are not readily assigned. Consequently, the records of these common and long-known species are not wholly reliable and the exact limits of their relative distributions is still uncertain.

Before separating the West Indian brittle-stars into the half-dozen groups into which the sea-stars were divided, we must eliminate the following 14 species, which are known from only a single locality and not infrequently from only a single specimen.

Ophiacantha oligacantha.
Ophiomitrella glabra.
Ophiophragmus filograneus.
pulcher.
Amphipholis pachybactra.
Amphiodia rhabdota.
trychna.
Amphiodia tymbara.
Amphioplus coniortodes.
thrombodes.
Ophiactis cyanosticta.
Ophiopsila vittata.
Ophioderma phœnium.
squamosissimum.

There are several other species which are so little known that they might perhaps well be included in this list, but as they are known from at least two different localities it seems fairer to include them in one of the following groups:

The first or tropicopolitan group includes only two species, each of which is small and well-adapted by its habits for transportation on the foul bottoms of vessels. It seems highly probable that their wide distribution is thus quite artificial and has no significance from the zoögeographical point of view. The two species are: *Amphipholis squamata* and *Ophiactis savignyi*. The first of these is really cosmopolitan, for it occurs far outside the tropics, both north and south.

Of the remaining 42 species, one-half are widely distributed in the tropical Atlantic and under favorable conditions their occurrence may be expected anywhere between South Carolina and Brazil. Only 5 of them are as yet recorded from north of Florida on the mainland coast, but 15 have been reported from the Bahamas and 14 from the Bermudas; 5 are already recorded from the eastern Atlantic, and when the littoral faunas of Ascension and the western coast of Africa are better known, it is probable that others will be found there; 18 are already reported from Brazil. These 22 tropical Atlantic species are the following:

Ophiomyxa flaccida.
Astrophyton muricatum.
Amphiura stimpsonii.
Hemipholis elongata.
Ophiostigma isacanthum.
Amphiodia planispina.
repens.
Ophiocnida scabriuscula.
Ophiothrix angulata.
suensonii.
Ophionereis reticulata.
Ophiocoma echinata.
pumila.
riisei.
Ophiopsila riisei.
Ophioderma appressum.
brevicaudum.
brevispinum.
cinereum.
Ophiozona impressa.
Ophiolepis elegans.
paucispina.

The line between the preceding group and that which I call the strictly West Indian is not a hard-and-fast one, but the following species are not known from south or east of Tobago and Trinidad and only two occur on the mainland coast north of Florida; one of these and a third species occur at Bermuda. Only one species in the group is known from the Bahamas. These 13 West Indian species are:

Amphiura vivipara.
Ophiophragmus lütkeni.
septus.
wurdemanii.
Ophionema intricata.
Amphipholis gracillima.
Amphiodia pulchella.
Ophiactis lymani.
Ophiothrix brachyactis.
œrstedii.
Ophionereis squamulosa.
Ophioderma guttatum.
rubicundum.

A number of species in the tropical Atlantic and West Indian groups need a word of comment. Of *Amphiura stimpsonii*, specimens in really

shallow water have not been taken south of St. Thomas. The distribution of *Hemipholis elongata* is remarkable, for the species seems to be common at Charleston (South Carolina) and has been taken in Florida, but it has not been met with in the West Indies proper, although it is recorded from Trinidad and Brazil. The occurrence of *Amphiodia planispina* at the Tortugas, Porto Rico, and Brazil seems to warrant placing it in the tropical Atlantic class. The occurrence of *Ophioderma brevispinum* in Buzzards Bay, Massachusetts, gives the characteristically West Indian genus *Ophioderma* a remarkable northern extension. The little species *Amphiura vivipara* is known as yet from only the Tortugas and Tobago, but it has just recently been described and is so small and so secretive in its habits that it has doubtless been overlooked elsewhere. The species of *Ophiophragmus* are still very imperfectly known; *lütkeni* (originally taken at St. Thomas) is common at Pigeon Point, Tobago, but is not known from elsewhere; *septus* (also originally from St. Thomas) is known now from off Cape Hatteras, in 52 fathoms, as well as from Tobago; *wurdemanii*, originally from South Carolina and Florida, is recorded from Trinidad. It seems to me quite probable that this last record is erroneous. The extraordinary *Ophionema intricata*, originally described from St. Thomas, is not rare at Sandy Point, Buccoo Bay, Tobago, but is not known from elsewhere. The records of *Amphiodia pulchella*, from Tortugas and St. Lucia, are indicative of a general West Indian range. Of *Ophiactis lymani*, we can only say that its small size and secretive habits are the probable reason for the scarcity of records, since it occurs at the Tortugas as well as at Bermuda and Tobago. The Tortugas and Tobago are the only known localities for *Ophiothrix brachyactis*, while *Ophionereis squamulosa* is common at both those places and is also known from St. Thomas. It is probable that the brittle-stars, recorded by me from Porto Rico (1901, Bull. U. S. Fish. Com., 2, p. 248) as *O. dubia*, are really *squamulosa*. The remarkable *Ophioderma guttatum*, originally described from St. Thomas, occurs along the north coast of Jamaica but is very rare, while it is common and reaches a large size on Buccoo Reef, Tobago; it is not known elsewhere.

There remain 7 species, which seem to represent different faunal elements from those as yet listed. Of these, only one is southern in its relationships. This is *Ophioderma januarii*, a Brazilian species, which is rare at Tobago. There is no species with a Mediterranean affiliation, though the occurrence of such characteristic West Indian genera as *Ophiopsila* and *Ophioderma* in the Mediterranean must not be overlooked.

The following 6 species may be grouped as northern in their distribution:

Amphiura palmeri.	Amphioplus abditus.
kukenthali.	Ophiothrix lineata.
Ophionephthys limicola.	Ophionereis olivacea.

Of these, *A. palmeri* is recorded from rather deep water from off Barbados, but none of the others is known from east or south of St. Thomas. The mud-loving *Amphioplus abditus*, ranging as far north as Woods Hole, Massachusetts, has not been found in the West Indies proper but occurs on the Florida coast and at the Tortugas. The only known stations for *A. kukenthali* are the Tortugas and St. Thomas. I did not find it at Tortugas and it is quite possible that it is not really a littoral species in the strict sense. The same remark is applicable to *Ophionephthys limicola*, which I dredged at the Tortugas, while St. Thomas is the type-locality. The well-marked and easily recognized *Ophiothrix lineata* is known only from Florida and the Tortugas, a remarkably restricted range for an *Ophiothrix*. The very rare *Ophionereis olivacea* is known from only two specimens, one from Porto Rico and one from Key West.

ECHINOIDEA. SEA-URCHINS.

The littoral echini of the West Indies are not numerous so far as species are concerned, but in number of individuals they are often excessively abundant. On and about the coral reefs, the dreaded poisonous "black sea-egg" (*Centrechinus antillarum*) is common and on certain areas it is so numerous that a person can scarcely move about without touching one. On many reefs the boring urchin (*Echinometra lucunter*) occurs actually by the thousands, and it is almost always common. On suitable grassy bottoms the "white sea-egg" (*Tripneustes esculenta*) is very common, and in similar localities *Lytechinus variegatus* may be so abundant that one can not walk on the bottom without crushing them under foot. Although *Eucidaris tribuloides* is often uncommon and hard to find, occasionally it occurs in great numbers and may be gathered literally by the bushel. These 5 urchins may be expected anywhere in the West Indian region in considerable numbers, if the bottom and water are suitable. None of the other echini in the following list are common except locally, but any one of them may prove abundant if a particular locality is suitable. Thus I have never found *Clypeaster rosaceus* common until 1917, when it proved to be abundant on the reef-flats at Bush and Bird Keys, Tortugas.

The following 18 species are the littoral sea-urchins of the West Indian region:

Eucidaris tribuloides (Lamarck).
Centrechinus antillarum (Philippi).
Arbacia punctulata (Lamarck).
Lytechinus variegatus (Leske).
Tripneustes esculentus (Leske).
Echinometra lucunter (L.).
viridis A. Agassiz.
Clypeaster rosaceus (L.).
subdepressus (Gray).
Encope emarginata (Leske).
michelini (Agassiz).
Mellita quinquiesperforata (Leske).
sexiesperforata (Leske).
Echinoneus cyclostomus (Leske).
Moira atropos (Lamarck).
Plagiobrissus grandis (Gmelin).
Meoma ventricosa (Lamarck).
Brissus brissus Leske.

None of the above species, when adult, is at all difficult to recognize nor is any one of them of doubtful authenticity. The two species of *Clypeaster* are quite unlike and the same is true of the *Encopes* and *Mellitas*. The two *Echinometras* are more liable to confusion because *E. lucunter* is so variable in form, length of spines, and color, but no one who has once seen typical *viridis* will have any difficulty. The cassidulid, *Rhynchopygus caribœarum* (Lamarck), is probably a littoral species, but it is so rare and little known that I have not ventured to include it in the above list. On the other hand, the spatangoid, *Schizaster orbignyanus* A. Agassiz, is probably not a littoral species, but I found a bare test under a rock in shallow water at Montego Bay, Jamaica, in March 1912, and it may possibly be truly littoral. It is, however, a very rare and little-known species.

Of the above littoral species, *Echinoneus cyclostomus* seems to be tropicopolitan; at least it is known from not only the West Indian region but throughout the Indo-Pacific from Mauritius to Hawaii and Easter Island.

Of the remaining 17 species, 9 have a general tropical Atlantic distribution from the Carolinas, or Florida at least, to Brazil. Of these, 5 are already known from the eastern Atlantic and at least two of the others will probably be found there. One of the 9 (*Brissus*) is not yet known from Brazil, but it almost certainly will be found there. These are the 9:

Eucidaris tribuloides.
Centrechinus antillarum.
Lytechinus variegatus.
Tripneustes esculentus.
Echinometra lucunter.
Clypeaster subdepressus.
Mellita quinquiesperforata.
sexiesperforata.
Brissus brissus.

It is a remarkable fact that there is not a single echinoid that can be called distinctively West Indian, unless possibly one or two of the following group, which seems to have a northern range, should prove to extend further south than is at present known. This northern group includes 7 of the remaining 8 species, as follows:

Arbacia punctulata.
Echinometra viridis.
Clypeaster rosaceus.
Encope michelini.
Moira atropos.
Plagiobrissus grandis.
Meoma ventricosa.

Of all these, *Arbacia* has the most peculiar distribution, for its range seems continuous from the Tortugas and Florida northward along the coast to southern Massachusetts. It does not occur at Bermuda nor is it known from the Bahamas, but it is recorded from both Cuba (northwestern coast only) and Hayti (an old record that needs verification). It does not occur at Jamaica, nor is it known from Porto Rico or the Lesser Antilles, but it does occur in Trinidad and Tobago. It occurs on the coast of Yucatan and has been reported

from Brazil; the latter record, however, is probably due to confusion with the Brazilian species, *Arbacia lixula* (L.). This distribution is quite incomprehensible, but comparison of specimens from Tobago, the Tortugas, and Massachusetts shows no reason to doubt that all are *punctulata*.

The range of *Echinometra viridis* is quite restricted, extending only from the Tortugas to St. Thomas, but *Clypeaster rosaceus* ranges northward along the coast to South Carolina, southward at least to Guadeloupe, and is common in the Bahamas. The range of *Encope michelini* is like that of *Echinaster tenuispinus*, from the Tortugas northward along the west coast of Florida and thence westward along the Gulf Coast to Mexico. The remarkable spatangoid *Moira* has a peculiar distribution, being common at Beaufort, North Carolina, but known also from Florida, Texas, Jamaica, St. Thomas, and Gaudeloupe. The finest of all spatangoids, *Plagiobrissus grandis*, is common near Nassau, Bahama Islands, but reliable records from elsewhere are rare; it is said to occur at Tampa, Florida; a fragment is known from the Tortugas, and there is a specimen recorded in the "Revision of the Echini" from Mexico. The range of *Meoma* is much greater, extending from Central America, Florida, the Bahamas, and Jamaica to Guadeloupe.

The only remaining echinoid, *Encope emarginata*, appears to have a rather southern range, occurring from Uruguay to Venezuela and even to Martinique. It is reported from Charleston, Florida Gulf Stream, Nicaragua, and Yucatan, but these records are old and indefinite and are probably erroneous.

HOLOTHURIOIDEA. HOLOTHURIANS.

The holothurians are, next to the brittle-stars, the most abundant of the littoral echinoderms of the West Indies, but as they are not easily preserved and as preserved material is not attractive or interesting in appearance, they are as yet very inadequately known. They can be accurately determined only by an examination of the calcareous particles in the skin, and the study of these often involves the high power of a microscope. Moreover, we know as yet little about the growth-changes in holothurians, particularly as regards these calcareous particles, and hence identifications made years ago are of doubtful validity, while many of those made to-day are merely tentative. Many common West Indian holothurians are as yet unnamed and very few are adequately described. The following list of 24 species includes all those named forms, which are recognizable with sufficient ease and certainty to make the records of their occurrence reasonably reliable. It may be of interest to mention that there are in the M. C. Z. collection more than 25 additional littoral species, chiefly from the Tortugas, Jamaica, and Tobago, which seem to be undescribed and

are as yet nameless. It is obvious, then, that our knolwedge of the West Indian littoral holothurians is as yet too fragmentary and unreliable to give value to any deductions with reference to their distribution. From the list here given I have excluded 3 of Selenka's species supposed to be from Florida, 4 species of other regions recorded from the West Indies, and several of Sluiter's recently described species concerning the status or littoral distribution of which I am still in doubt. The 24 holothurians here accepted are the following:

Euapta lappa (Müller).
Synaptula hydriformis (Lesueur).
Leptosynapta acanthia (H. L Clark).
inhærens (O. F. M.).
roseola Verrill.
Chiridota rotifera (Pourtales).
Cucumaria punctata Ludwig.
Thyone briareus (Lesson).
fusus (O. F. M.)?
gemmata (Pourtales).
suspecta Ludwig.
Psolidium braziliense (Theel).
Holothuria captiva Ludwig.
cubana Ludwig.
densipedes H. L. Clark.
floridana Pourtales.
glaberrima Selenka.
grisea Selenka.
impatiens (Forskaal).
rathbuni Lampert.
surinamensis Ludwig.
Stichopus moebii Semper.
Actinopyga agassizii (Selenka).
parvula (Selenka).

Of the above species, *Leptosynapta acanthia* and *Holothuria densipedes* are known each from only one locality and hence may be eliminated from any discussion of distribution. The species listed as *Thyone fusus* may also be ignored, for while it was not rare in Buccoo Bay, Tobago, the specimens taken were all very small, and it is quite improbable that they are really identical with the European *fusus*. Perhaps the same should be said of *Psolidium braziliense*, which occurred in coralline algæ at Buccoo Bay, with the *Thyone*. None of the specimens taken was nearly large enough to make its identity certain.

Of the remaining 20 species, *Holothuria impatiens* and *Actinopyga parvula* seem to have a tropicopolitan distribution, but a critical study needs to be made to ascertain it precisely

There are six species which may be called tropical Atlantic, as their range extends from Bermuda or South Carolina to Brazil. They are:

Synaptula hydriformis.
Chiridota rotifera.
Thyone gemmata.
Holothuria grisea.
rathbuni.
surinamensis.

The first two of these are unmistakable and there is no doubt about their range, but the *Thyone* is by no means unmistakable and it is not certain that the Carolinian and Brazilian records refer to the same species. The status of *H. grisea* is somewhat uncertain, as it is very possibly only the young of *H. floridana;* it is recorded from the eastern Atlantic as well as from Brazil. Both of the other holothurias are well-characterized species and certainly occur at Bermuda, while they

are recorded from Brazil and five or six intermediate places. None of the 6 species, except *H. grisea*, is known from the eastern Atlantic.

There are no fewer than 7 holothurians which seem to be characteristic of the West Indies; these are:

Euapta lappa.	Holothuria floridana.
Cucumaria punctata.	glaberrima.
Thyone suspecta.	Actinopyga agassizii.
Holothuria captiva.[1]	

Of these, *Euapta lappa* is particularly notable for its large size and striking appearance, which prevent its being overlooked; it is not known from Bermuda, nor from north of southern Florida, nor from the Gulf of Mexico, northern South America, or any point south or east of Tobago. The *Thyone* is known only from Jamaica and Barbados. Both the *Cucumaria* and the *Actinopyga*, as well as *H. captiva*, are known from Bermuda, although the *Actinopyga* is very rare there and is possibly only accidental. Both *Holothuria floridana* and *H. glaberrima*, which have not been found at Bermuda, are known from the Bahamas to Barbados. The remaining 5 holothurians are all forms whose distribution is more or less distinctly northern:

Leptosynapta inhærens.	Holothuria cubana.
roseola.	Stichopus mœbii.
Thyone briareus.	

The two species of *Leptosynapta* are not actually known from any point south of Bermuda, and the occurrence of *inhærens* there is known only from a single specimen, which may perhaps have been a young *acanthia*. The well-known *Thyone briareus* occurs along the American coast from Texas to Massachusetts; specimens of *Thyone* in the M. C. Z. collection from Porto Seguro, Brazil, have been identified as *briareus*, but as their preservation is poor it is quite possible they are not that species. Little is known of *H. cubana*, but *Stichopus mœbii* is a very abundant species in Bermuda, Florida, and Jamaica. It does not occur at Tobago and there are no records for it south of Antigua.

[1]There is strong evidence in support of the opinion that *Holothuria captiva* is identical with *Actinopyga parvula*.

II. LITTORAL ECHINODERM FAUNA OF WEST INDIAN ISLANDS AND ADJACENT REGIONS.

In the table herewith published showing the littoral echinoderme known from each island, 116 species are listed. One has but to glance at the table to see how few areas there are where the echinoderms are even superficially known. Almost nothing is known of the coast between New Orleans and Vera Cruz or of that much more extensive, varied, and important region between Vera Cruz and Colon. That which is known of the Vera Cruz fauna is merely tantalizing. Although there are many Brazilian records, at no point between Colon and Rio Janeiro has there been any attempt to make a collection of echinoderms. It is only fair to say, therefore, that we know almost nothing of the littoral echinoderm fauna of the eastern coast of tropical and subtropical America. When we turn to the islands themselves, conditions are somewhat better, but we know absolutely nothing of the Caymans' marine fauna, nor of that of the many islands and islets in the western part of the Carribean Sea, except for half a dozen species from Swan Island. In the Bahamas no one place has been very completely explored and many records do not designate the particular island. It is, therefore, necessary to place all Bahaman records under a single head. Very little is known of the littoral echinoderms of Haiti and San Domingo except for Dr. Weinland's collection of many years ago; of the Lesser Antilles, not a single one has been thoroughly explored.

There are, however, half a dozen islands, besides the mainland coast of Florida, where more than one-third of the 116 species have been taken and to each of these areas a few remarks are due.

FLORIDA.

From the coast of Florida, 69 species are known, a larger number than from any island except the Tortugas. But it must be remembered that 3 species, *Echinaster spinulosus*, *Ophiophragmus filograneus*, *Encope michelini*, represent a distinctly Gulf Coast fauna, and the distribution of 5 other species is so local or so peculiar as to make their occurrence of special note. These are: *Amphioplus coniortodes*, *A. thrombodes*, *Ophiothrix lineata*, *Arbacia punctulata*, and *Plagiobrissus grandis*.

Of the 69 species, the following 13 are known from Florida and not from the Tortugas, but in many cases unfortunately we do not know from just what part of the Florida coast they come.

Luidia senegalensis.
Stegnaster wesseli.
Hemipholis elongata.
Ophiophragmus filograneus.
wurdemanii.
Amphioplus coniortodes.
thrombodes.
Ophionereis olivacea.
Mellita quinquiesperforata.
Moira atropos.
Thyone briareus.
Holothuria cubana.
surinamensis.

The absence of suitable bottoms at the Tortugas undoubtedly accounts for the absence of some of these, as *Luidia senegalensis*, *Moira atropos*, and *Thyone briareus;* but it is probable that most of them will ultimately be found there, when our knowledge is more complete. It is noteworthy that of the 69 Florida species, 32 (or almost one-half) are not known from Tobago, while 20 are as yet unrecorded from Jamaica and 27 are not listed from St. Thomas. While our knowledge is as yet too imperfect to make deductions very safe, the increasing difference in the faunas as the distance from Florida increases is so obvious and so regular that it can not be overlooked.

One other feature of the Florida fauna must be mentioned. More than a third (24) of the species occur on the coast north of Florida, but not one of these has peninsula Florida as the southern limit of its range and only one, *Amphioplus abditus*, reaches its southern limit at the Tortugas. At least one echinoderm, *Asterias forbesii* (Desor) occurs on the coast of Florida, finding the southern limit of its range there, but as this species does not occur at Tortugas or Bermuda and is a distinctly northern species, it is not included in the tables.

THE TORTUGAS.

No fewer than 76 littoral echinoderms occur at the Tortugas. Perhaps 4 or 5 of these are not so strictly littoral as my restrictions require, but there are certainly more than 70 species which may be collected at the Tortugas by hand, without trawl or dredge. Thus the seat of the Carnegie Laboratory is apparently the best place in the West Indies for this particular sort of fauna. Four of the species are as yet known only locally and 6 are tropicopolitan, 35 are of the Tropical Atlantic group, 13 are distinctly West Indian, and 18 are northern. There are 56 species that the Tortugas have in common with Florida, 52 in common with Jamaica, 47 with St. Thomas, and 45 with Tobago. Only 19 species, just one-fourth, occur on the mainland coast north of Florida.

BERMUDA.

The echinoderm fauna of Bermuda has been quite thoroughly collected and studied during the past 30 years and is probably better known to-day than that of any other area in the region under consideration. There are 42 species here listed which occur there, and there is at least one unidentified holothurian not included herein. Of the 42 species, 1 is endemic and 4 are tropicopolitan, 4 are northern, 6 are West Indian, 1 is Mediterranean, and all the rest (26) have a wide distribution in the tropical Atlantic. It is rather remarkable that the ophiuran fauna is somewhat scanty, without an endemic species. Both at the Tortugas and Tobago more than half of the echinoderms are brittle-stars, while at Bermuda they comprise less than 43 per cent. Of the 42 echinoderms found at Bermuda, 34 occur at the Tortugas, 34 at Jamaica, 32 at Tobago, 26 at St. Thomas,

and 26 on the coasts of peninsular Florida. It is notable that all of the echini and brittle-stars occurring at Bermuda are found also at Tobago. It would be hard to bring out more clearly how distinctively West Indian the echinoderm fauna of Bermuda is. The only non-West-Indian elements in it are the sea-star, *Coscinasterias tenuispina*, of the Mediterranean, which was possibly introduced by means of ship-bottoms, and the northern synaptids, whose occurrence is difficult to explain.

JAMAICA.

The echinoderm fauna of Jamaica is rich and varied, including 62 species of the present list and more than a dozen as yet unidentified holothurians. Indeed, the holothurians form a very conspicuous feature of the fauna on the reefs and in shallow water. Intensive collecting of echinoderms has been carried on at three widely separated points on the Jamaican coast: Montego Bay and Port Antonio near the western and eastern ends respectively of the northern coast, and in the vicinity of Port Royal on the southern coast. The last is much the best region, the so-called "lakes" at Port Royal, the rocky coast across the harbor entrance and the outside cays, particularly Drunkenman Cay, affording a diversity of habitats that is very productive. Both Port Antonio and Montego Bay yielded species not taken elsewhere, but it is quite probable that they will be found in the Port Royal region when it is fully explored.

Of the 62 species, not a single one is endemic. More than half (33) belong to the general tropical Atlantic fauna, while 14 are characteristically West Indian. There are 6 tropicopolitan forms. Only a single species is indicative of southern affinities, but 8 are plainly northern. There are 52 species in common with the Tortugas, 47 in common with St. Thomas, and 42 in common with Tobago, but only 34 in common with Bermuda. More sea-stars and more holothurians are known from Jamaica than from any other place, but the number of brittle-stars is small, there being five other areas from which more brittle-stars are known. There is little doubt that the number of mud-inhabiting ophiurans known from Jamaica will be considerably increased by further collecting in suitable areas.

PORTO RICO.

The echinoderm fauna of Porto Rico is not rich either in number of species or (in most places) in number of individuals. It is remarkably like that of Jamaica, all of the sea-stars, all the echini, and all but one of the holothurians being common to the two islands, while all of the 6 brittle-stars recorded from Porto Rico but not yet known from Jamaica are mud-loving species which will very probably be found in suitable localities at the British island. With both St. Thomas and the Tortugas, Porto Rico has 43 species in common but with Tobago only 33 and with Bermuda only 28. Of the Bermudan

fauna, however, just two-thirds (66 per cent) occurs at Porto Rico, while of the Tobagoan fauna only a little more than half (53 per cent) is found there.

Of the 54 species, 2 are as yet known only from Porto Rico; 29 are common throughout the tropical Atlantic and 11 others are distinctively West Indian; 4 are tropicopolitan and 7 have a northern range; only 1 can be considered representative of a southern fauna. The number of sea-stars known from Porto Rico is exceptionally large, nearly one-fifth of the echinoderms belonging in that class, whereas only 12 per cent of the entire West Indian list is made up of sea-stars.

ST. THOMAS.

The United States Virgin Islands, so long known as the Danish West Indies, are classic ground for the student of echinoderms, as a very large proportion of the West Indian species were first recorded from there, thanks to the industry and great abilities of the celebrated Danish zoölogist, Lütken. In the present paper, I have not attempted to keep separate the records from the different islands, but have included them all under "St. Thomas," since the name Danish West Indies is no longer correct and the recently coined official name for the group is also open to misunderstanding.

None of the 56 species here listed from St. Thomas is endemic, but on the other hand 6 are tropicopolitan. There are 28 tropical Atlantic and 13 distinctly West Indian forms. The remaining 8 species all have northern affiliations. There are 44 species which occur in Porto Rico, or 81 per cent of that island's fauna; 47 which are found in Jamaica, 75 per cent of that fauna; 27 which are known at Bermuda, 64 per cent of that fauna; 48 which occur at the Tortugas, 63 per cent of that fauna; and only 38 which are found at Tobago, just 60 per cent of that fauna.

TOBAGO.

The echinoderm fauna of Tobago is largely confined to the vicinity of Pigeon Point on the southwestern part of the island, where extensive coral reefs protect the shallow waters of Buccoo Bay. Although we made brief visits to several points on the southeastern and northeastern sides of the island, we found very few echinoderms indeed in those places. But in Buccoo Bay and on Buccoo Reef, there is an exceedingly rich fauna, especially of brittle-stars, which constitute nearly 60 per cent of it.

The most notable member of this fauna is the comatulid, *Tropiometra carinata*, a conspicuous representative of a southern fauna, common on the coast of Brazil. Two other representatives are noteworthy—the little bright rose-colored holothurian *Psolidium braziliense* and the handsome brittle-star *Ophioderma januarii*. The latter is apparently rare at Tobago, but a number of specimens of *Psolidium* were taken, though all are very small.

Besides these 3 southern species, not found elsewhere in the West Indies, 7 other species are not yet known from any place but Tobago. One of these is the little *Thyone*, referred to on page 63, but the others are all brittle-stars. Of these, two are ophiodermas and deserve special attention. One, *Ophioderma squamosissimum*, has long been known from the unique holotype in the Copenhagen Museum, which is from an unknown locality in the West Indies, almost certainly not Tobago and very possibly St. Thomas. This brilliantly colored brittle-star (plate 3, fig. 2) is rare at Tobago, only 5 specimens being found on Buccoo Reef at extreme low-tide. None is as large as the holotype. The other notable *Ophioderma* at Tobago is *O. phœnium* H. L. Clark (1918, Bull. M. C. Z., 62, p. 333), which seems to be a fairly common, endemic species. The coloration is conspicuous, sometimes all green, sometimes all red, but usually a red disk with green arms (plate 3, fig. 1). Another remarkable *Ophioderma*, *O. guttatum*, is common on Buccoo Reef and reaches a large size there. It is possible that this is a southern species, for while it was described from a single specimen taken at St. Thomas, and I have taken it twice in Jamaica, these three specimens are all small, only about half as large as the adults of Tobago. Associated with the ophiodermas on Buccoo Reef were great numbers of *Ophiomyxa flaccida*, of very diverse hues; olive-green either with or without white markings is a usual color for this species, but olive-yellow, passing into brilliant yellow (plate 1, fig. 2) or brown passing through red-brown into red of various shades (plate 1, fig. 1) are common. Another brilliant brittle-star found on Buccoo Reef was the unique type-specimen of *Ophiothrix œrstedii* var. *lutea* H. L. Clark (1918, Bull. M. C. Z., 62, p. 314), whose bright orange coloration (plate 2) is very distinctive.[1]

All of the half-dozen tropicopolitan echinoderms of the West Indies are common at Tobago and there are two species which have northern rather than southern relationships. One of these is the sea-urchin, *Arbacia punctulata*, whose distribution from Tobago to Massachusetts (along the mainland coast?) is so puzzling. Of the remaining 45 species, three-fifths are typically West Indian.

No fewer than 32 species are common to Bermuda and Tobago; this is 76 per cent of the Bermudan fauna and is a very remarkable fact. It can be explained only on the ground that Tobago is the home of 45 tropical Atlantic and West Indian species, and it is from this wide-ranging group that the Bermudan littoral echinoderm fauna has been almost wholly derived. Of the Jamaican fauna, 43 species (69 per cent) occur at Tobago, and of the St. Thomas fauna, 38 (68 per cent). There are 45 species common to Tobago and the Tortugas, but this is only 59 per cent of the Tortugas fauna.

[1] I am indebted to Dr. Mayor for making colored sketches from living specimens of these brilliant ophiurans. From these sketches and the preserved specimens, Mr. J. Henry Blake has made the beautiful drawings reproduced herewith.

III. CONCLUSIONS.

As already stated, the data at present available are too fragmentary to warrant any reliable deductions. We can as yet scarcely guess at the origin of the littoral fauna of the West Indian Islands, but certain things are suggested by this study which may be mentioned as requiring further consideration:

1. There is no very close relationship with the Mediterranean fauna. Of the 55 genera concerned, only 25 occur in the Mediterranean, and only one of these is as yet unknown on the western coast of America.

2. There is a notable resemblance to the fauna of the western coast of tropical America, four-fifths of the genera (44) being known to occur there. And in many genera specific differences between the West Indian and West Coast forms are very slight.

3. The fauna of the Bermudas is practically all derived from the West Indies, and so recently that no endemic species have as yet arisen. The only endemic species in Bermuda is *Leptosynapta acanthia*, which is probably derived from one of the northern members of the genus.

4. The fauna of Tobago unquestionably contains a southern element derived from the Brazilian coast.

5. If we assume that the genus *Arbacia* arose on the western coast of America, the present distribution of the genus and of the species *punctulata* can be explained as follows: The Caribbean Sea was at one time an eastward extension, a narrow-mouthed gulf of the eastern Pacific ocean, formed after *Arbacia* was well distributed north and south of the present isthmus of Panama. The present species *punctulata* entered this gulf on both shores and followed them eastward. After the closing of the gulf and the formation of the Lesser Antilles, the species being exclusively littoral spread, not in all directions, but only northward along the Mexican and United States coasts and eastward along the South American coast to Trinidad and Tobago. Later the genus passed southward to Brazil and eastward to Africa and the Mediterranean in the form of *lixula*, which may well have been derived from *punctulata*. Along the coast of the United States, the local conditions have not been favorable to developing a new species. From Florida, *punctulata* has crossed over to Cuba and perhaps extended along the north shore of that island to Haiti. According to this theory, *Arbacia* does not and never has occurred in the Lesser Antilles nor in Jamaica and probably does not occur on the south side of Cuba or in Porto Rico. It may be found in the Bahamas. It certainly ought to occur in favorable places along the north coast of South America. Until it can be shown to occur there, the hypothesis here proposed with reference to *Arbacia* lacks adequate foundation. But the distribution of *Hemipholis*, *Echinaster*, *Moira*, *Meoma*, *Thyone*, *Stichopus*, and similar genera, as shown in the table on pages 71-73, gives some support to the theory and warrants its consideration in interpreting the West Indian fauna.

Table showing distribution.

Name.	Bermuda.	Bahamas.	U. S. Coast, north of Florida.	Florida.	Tortugas.	Gulf Coast of United States.	Cuba.	Jamaica.	Haiti.	Porto Rico.	St. Thomas.	St. Bartholomew.	St. Christopher.	Antigua.	Montserrat.	Guadeloupe.	Dominica.	Martinique.	St. Lucia.	Barbados.	St. Vincent.	Grenada.	Tobago.	Trinidad.	N. Coast of S. A.	E. Coast of C. A. and Mexico.	Swan Island.	Brazil.	Eastern Atlantic.	Indo-Paoific.
Comatulida.																														
Nemaster iowensis																														
Tropiometra carinata																														
Asterodea.																														
Astropecten articulatus																														
duplicatus																														
Luidia alternata																														
clathrata																														
senegalensis																														
Oreaster reticulatus																														
Asterina folium																														
minuta																														
Stegnaster wesseli																														
Ophidiaster guildingii																														
Linckia guildingii																														
Echinaster sentus																														
spinulosus																														
Coscinasterias tenuispina																														
Ophiuroidea.																														
Ophiomyxa flaccida																														
Astrophyton muricatum																														
Ophiacantha oligacantha																														
Ophiomitrella glabra																														
Amphiura kukenthali																														
palmeri																														
stimpsonii																														
vivipara																														
Hemipholis elongata																														
Ophiophragmus filograneus																														
litkeni																														
pulcher																														
septus																														
wurdemanii																														
Ophionephthys limicola																														
Ophionema intricata																														
Amphipholis gracillima																														
pachybactra																														
squamata																														
Ophiostigma isacanthum																														

\+ indicates that an authentic specimen has been examined. – indicates a reliable record. ? indicates a doubtful record.

Table showing distribution—continued.

Name.	Bermuda.	Bahamas.	U. S. Coast, north of Florida.	Florida.	Tortugas.	Gulf Coast of United States.	Cuba.	Jamaica.	Haiti.	Porto Rico.	St. Thomas.	St. Bartholomew.	St. Christopher.	Antigua.	Montserrat.	Guadeloupe.	Dominica.	Martinique.	St. Lucia.	Barbados.	St. Vincent.	Grenada.	Tobago.	Trinidad.	N. Coast of S. A.	E. Coast of C. A. and Mexico.	Swan Island.	Brazil.	Eastern Atlantic.	Indo-Pacific.
Ophiuroidea—continued.																														
Amphiodia planispina	...	...	...	...	+	...	...	...	...	?	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
pulchella	...	...	...	...	+	...	...	...	...	?	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
repens	+	...	...	+	+	...	...	+	...	...	...	...	...	...	...	...	...	...	...	...	...	...	+	...	...	...	+	...	...	...
rhabdota	...	...	...	...	+	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
trychna	...	...	...	...	+	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
tymbara	...	...	...	...	+	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Ophiocnida scabriuscula	...	...	...	+	+	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Amphioplus abditus	...	...	+	+	+	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
coniortodes	...	...	...	+	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
thrombodes	...	...	...	+	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Ophiactis cyanosticta	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
lymani	+	...	...	+	+	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	+	...	...	...	...	...	...	...
savignyi	+	...	...	+	+	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	+	...	...	...	...	...	...	...
Ophiothrix angulata	−	−	+	+	+	...	+	+	+	−	+	...	...	...	...	...	...	...	−	−	...	...	+	−	−	+	...	+	...	...
brachyactis	...	...	...	...	+	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
lineata	...	...	...	+	+	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
œrstedii	...	−	...	+	+	...	−	+	...	−	+	...	...	...	...	...	...	...	...	−	...	...	+	...	+	+	...	...	...	...
suensonii	+	+	...	+	+	...	+	+	+	−	...	...	...	...	...	...	...	...	...	−	...	...	+	...	+	...	...	...	...	...
Ophionereis olivacea	...	...	...	+	+	...	...	...	...	?	...	...	...	...	...	...	...	...	...	...	...	...	+	...	...	...	...	...	...	...
reticulata	+	+	...	+	+	...	−	+	...	−	+	...	...	...	...	...	...	...	...	−	...	...	+	...	−	...	...	...	...	...
squamulosa	...	...	...	...	+	...	...	...	...	−	+	...	...	...	...	...	...	...	...	...	...	...	+	...	...	...	...	...	...	...
Ophiocoma echinata	+	+	...	+	+	...	...	+	...	−	+	...	...	...	...	...	...	−	...	...	...	...	+	...	...	...	...	...	...	...
pumila	+	−	...	+	+	...	+	+	+	−	+	...	...	...	...	−	...	...	...	−	...	...	+	...	...	+	...	...	...	...
riisei	+	+	...	+	+	...	...	+	+	−	+	...	...	...	...	−	...	...	...	−	...	...	+	...	...	...	...	...	...	...
Ophiopsila riisei	...	+	...	+	+	...	...	...	+	−	+	...	...	...	...	...	...	...	...	−	...	...	+	...	+	...	...	...	...	...
vittata	...	...	...	...	+	...	...	...	...	−	+	...	...	...	...	...	...	...	...	−	...	...	+	...	...	...	...	...	...	...
Ophioderma appressum	+	+	+	+	+	...	...	+	...	−	+	...	...	...	...	+	...	...	...	−	...	...	+	−	+	...	...	...	...	...
brevicaudum	−	+	...	...	+	...	...	+	...	−	+	...	...	...	...	...	...	...	...	−	...	...	+	...	?	...	...	...	...	...
brevispinum	...	...	+	+	+	...	+	+	+	−	+	...	...	...	...	−	...	...	...	−	...	...	+	...	−	...	...	...	...	...
cinereum	−	+	...	+	+	...	+	+	+	−	+	...	...	...	...	...	...	...	−	−	...	...	+	...	−	+	...	...	...	...
guttatum	...	...	...	...	...	...	...	+	...	...	...	...	...	...	...	...	...	...	...	...	...	...	+	...	...	...	...	...	...	...
januarii	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	+	...	...	...	...	...	...	...
phœnium	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	+	...	...	...	...	...	...	...
rubicundum	...	+	...	+	−	...	...	−	...	−	+	...	...	...	...	−	...	...	...	...	...	...	+	...	...	...	...	...	...	...
squamosissimum	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	+	...	...	...	...	...	...	...
Ophiozona impressa	+	...	...	...	...	...	+	+	...	−	+	...	...	...	...	+	...	...	...	−	...	...	+	...	−	...	...	...	...	...
Ophiolepis elegans	...	...	+	+	+	...	+	+	...	−	+	...	...	...	...	...	...	...	...	...	...	...	+	?	...	+	...	...	...	...
paucispina	+	...	...	+	+	...	+	+	...	−	+	...	...	...	...	...	...	...	...	...	...	...	+	?	...	...	...	...	...	...
Echinoidea.																														
Eucadaris tribuloides	+	+	−	+	+	....	+	+	+	−	+	...	...	−	...	...	...	...	−	−	...	...	+	...	−	−	+	+	...	...
Centrechinus antillarum	+	+	....	+	+	....	+	+	+	−	+	...	...	−	...	...	...	...	...	−	...	...	+	...	−	+	+	+	...	...

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Table showing distribution—continued.

Name.	Bermuda.	Bahamas.	U. S. Coast, north of Florida.	Florida.	Tortugas.	Gulf Coast of United States.	Cuba.	Jamaica.	Haiti.	Porto Rico	St. Thomas.	St. Bartholomew.	St. Christopher	Antigua.	Montserrat.	Guadaloupe.	Dominica.	Martinique.	St. Lucia.	Barbados.	St. Vincent	Grenada.	Tobago.	Trinidad.	N. Coast of S. A.	E. Coast of C. A. and Mexico.	Swan Island.	Brazil.	Eastern Atlantic.	Indo-Pacific.
Echinoidea—continued.																														
Arbacia punctulata	+	+	+	+	+	+	+	+	~	+	+											+								
Lytechinus variegatus	+	+	+	+	+		+	+	+	+	+											+								
Tripneustes esculentus	+	+	+	+	+		+	+	+	+	+											+								
Echinometra lucunter	+	+	+	+	+		+	+	+	+	+											+								
viridis																														
Clypeaster rosaceus																														
subdepressus																														
Encope emarginata																														
michelini																														
Mellita quinquiesperforata	+		+	+	+	+	+	+	+	+	+	+		+																
sexiesperforata																														
Echinoneus cyclostomus	+			+	+		+	+	+	+	+		+																	
Moira atropos																														
Plagiobrissus grandis																														
Meoma ventricosa	+	+	+	+	+	+	+	+	+	+	+	+		+																
Brissus brissus	+	+	+	+	+	+	+	+	+	+	+	+		+																
Holothurioidea.																														
Euapta lappa	+				+			+																						
Synaptula hydriformis	++	+	++	++	+			++																						
Leptosynapta acanthia	+				+																									
inhærens	+		+	+																										
roseola	+		++																											
Chiridota rotifera	+			+	+			+																						
Cucumaria punctata	+	+	+	+	+																									
Thyone briareus	+		+	+	+			+																						
fusus?	+																													
gemmata	+		+	+	+			+																						
suspecta																														
Psolidium braziliense					+			+																						
Holothuria captiva	+			+	+			+			+																			
cubana							+																							
densipedes																														
floridana	+			+	+		++																							
glaberrima									+																					
grisea																											?			
impatiens	+		++	++	+			+																						
rathbuni																														
surinamensis	+			+	+			+																						
Stichopus mœbii																														
Actinopyga agassizii	+								+																					+
parvula																														

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EXPLANATION OF PLATES.

PLATE 1.

FIG. 1. Ophiomyxa flaccida (Say). Red form. Buccoo Reef, Tobago. Natural size.
FIG. 2. Ophiomyxa flaccida (Say). Yellow Form. Buccoo Reef, Tobago. Natural size.

PLATE 2.

Ophiothrix œrstedii var. lutea H. L. Clark. Buccoo Reef, Tobago. Natural size

PLATE 3.

FIG. 1. Ophioderma phœnium H. L. Clark. Buccoo Reef, Tobago. Natural size.
FIG. 2. Ophioderma squamosissimum Lutken. Buccoo Reef, Tobago. Natural size.

HELIOTYPE CO. BOSTON.

Ophiomyxa flaccida (Say). Buccoo Reef, Tobago. Natural size.
FIG. 1, red form. FIG. 2, yellow form.

HELIOTYPE CO. BOSTON.

Ophothrix œrstedii var. *lutea* H. L. Clark. Buccoo Reef, Tobago. Natural size.

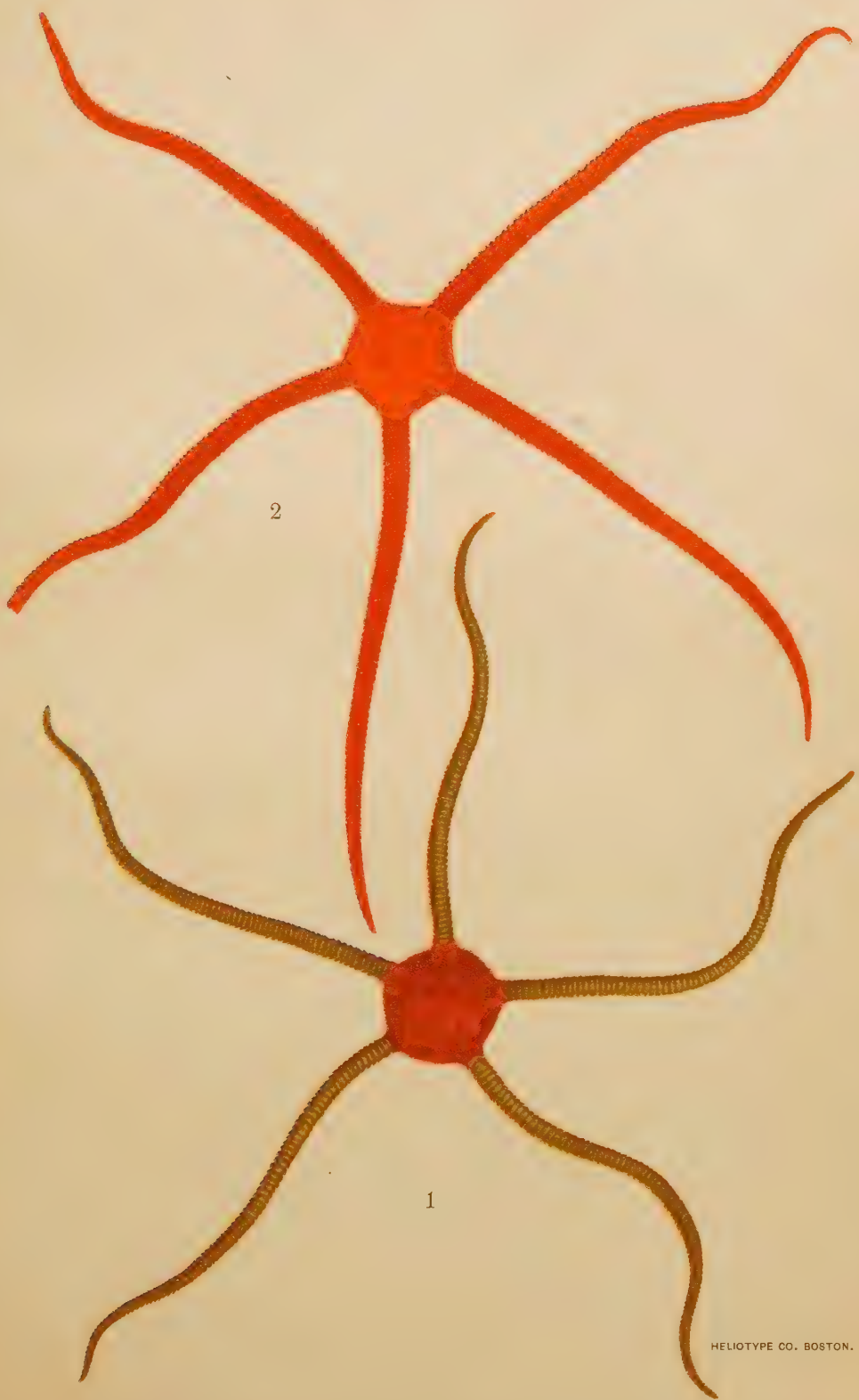

Fig. 1. *Ophioderma phoenium* H. L. Clark. Buccoo Reef, Tobago. Natural size.
Fig. 2. *Ophioderma squamosissimum* Lutken. Buccoo Reef, Tobago. Natural size.

IV.

FURTHER STUDIES ON THE CHEMISTRY OF LIGHT PRODUCTION IN LUMINOUS ORGANISMS.

By E. NEWTON HARVEY,
Of Princeton University.

FURTHER STUDIES ON THE CHEMISTRY OF LIGHT PRODUCTION IN LUMINOUS ORGANISMS.

By E. Newton Harvey.

The results embodied in this paper are the outcome of experiments made upon the dried luminous organs of a small ostracod crustacean, *Cypridina hilgendorfii*, abundant in the coastal waters of Japan. The structure of the luminous cells has been well described by Dahlgren in the Journal of the Franklin Institute for June 1916, and by Yatsu (Jour. Morph., vol. 29, p. 435, 1917). These cells contain the luminous substances concerned in the production of light, which are projected into the sea-water as a luminous secretion. This paper deals particularly with the chemistry of the luminous reaction. It will be considered in two sections, viz: I, reversibility of the photogenic reaction in *Cypridina*. II, the chemical nature of *Cypridina* luciferin and *Cypridina* luciferase.

Much of the work was performed in the Zoological Laboratory, Imperial University, Tokyo, Japan, and it gives me pleasure to acknowledge the kindnesses of Professors Ijuma, Yatsu, Watase, and Goto during my stay at the university. I am also deeply indebted to Professor C. Ishikawa, of the Agricultural College, Tokyo, for much assistance in collecting material, and I express my sincere thanks for his interest in my work.

I. REVERSIBILITY OF THE PHOTOGENIC REACTION IN CYPRIDINA.

In a previous paper on the question of animal luminescence[1] I have described two photogenic substances in *Cypridina hilgendorfii*, which I called photogenin and photophelein. Photogenin is destroyed below the boiling-point, is non-dialyzable, and is prepared by making a cold-water extract of the luminous animal and allowing it to stand in the air until no more light appears on shaking. This indicates that one of the photogenic substances, photophelein, has disappeared, leaving the photogenin. Photophelein is not destroyed by short boiling and will dialyze. It is prepared by making a hot-water extract of the luminous animal. The hot water destroys the photogenin, leaving the photophelein. Whenever two such solutions are mixed light appears.

On the grounds of method of preparation, relation to temperature, and dialysis, I regarded photogenin as comparable to luciferase and

[1]Harvey, E. N., *Am. J. Physiol.*, 1917, xlii, 318; also, The Chemistry of Light Production in Luminous Organisms, Carnegie Inst. Wash. Pub. No. 251, pp. 171–234, 1917.

photophelein as comparable to luciferin, two photogenic substances described by Dubois[1] in the beetle *Pyrophorus noctilucans* and in the mollusk *Pholas dactylus*. Dubois believes that luciferase is an oxidizing enzyme which oxidizes luciferin, an oxidizable substance, with light production. Neither luciferase nor luciferin alone in solution can produce light, but light appears if solutions of the two are mixed and it continues as long as any luciferin remains unoxidized. Dubois has also been able to produce light by oxidizing luciferin (alone) with a small crystal of $KMnO_4$, by H_2O_2 (with or without blood containing hemoglobin), BaO_2, PbO_2, and other oxidizing agents. Through the kindness of Professor Dubois, I have received some material of *Pholas dactylus* preserved in sugar and I can confirm his results on the effect of $KMnO_4$ and other oxidizing agents in producing light with luciferin of *Pholas*. I have likewise repeated my own experiments with the photophelein of *Cypridina*, using a whole series of oxidizing agents applied in the same way as with the luciferin of *Pholas*, and, as previously, have failed to obtain any light with this substance.[2] The difference in our results is, therefore, not to be referred to a difference in method of experiment but to a difference in the animals themselves.

I found also that if one takes a concentrated solution of photogenin, filtered through a porcelain or silicious filter candle to remove all granules and cell fragments, and adds to it a little saponin powder or amyl alcohol or NaCl or other inorganic salt crystals or tissue extracts of certain invertebrate non-luminous animals, that light would appear. Because NaCl could not possibly be oxidized by photogenin (=luciferase), or any other substance, and because of my inability to make photophelein (=luciferin) luminesce with oxidizing agents, I regarded the photophelein itself as the source of the light and the oxidizable body. I have compared photogenin to zymase and photophelein to the co-enzyme of zymase, believing that we are dealing with a system similar to that of the enzyme—co-enzyme system of yeast. Hence the name photophelein or body assisting in the production of light.

I now believe that under the term photophelein I have previously included two separate substances. One of these is the thermostable dialyzing substance extracted from *Cypridina* by hot water. Although this substance can not be oxidized with light production by oxidizing

[1] Dubois, R., *Compt. rend. Soc. Biol.*, 1885, xxxvii, 559.

[2] The following oxidizing agents (added, where possible, in minute crystal or powder form) all gave light with *Pholas* luciferin, but no light with *Cypridina* luciferin: $KMnO_4$, $K_2Cr_2O_7$, PbO_2, Na_2O_2, BaO_2, MnO_2, $K_3Fe(CN)_6$, $K_2S_2O_8$, $Na_2B_4O_3$, and H_2O_2. The following oxidizing agents gave no light with either *Pholas* luciferin or *Cypridina* luciferin: K_2CrO_4, CrO_3, $KClO_3$, $KClO_4$, $FeCl_3$, KNO_3, Cl or Br water, I in KI, Na hypochlorite, hypobromite, or hypoiodite, colloidal Ag or Pt, benzoyl peroxide, potato or turnip juice, or blood containing hemoglobin or hemocyanin. If H_2O_2 in addition to the oxidizing agent is added to *Cypridina* luciferin, no light appears except a faint momentary flash with Na hypochlorite and hypobromite. As this faint flash also appears with thoroughly boiled extracts of *Cypridina*, lacking luciferin, it can have no significance. If H_2O_2 in addition to the oxidizing agent is added to *Pholas* luciferin, the light is in some cases brighter than with H_2O_2 alone.

agents, it does oxidize spontaneously (also without light production) in the air and loses its power of producing light with photogenin. In the absence of air its solutions are stable for months. Once oxidized, it can again be reduced and will again give light if photogenin is added. It is therefore an oxidizable material and, I believe, similar to the luciferin of *Pholas*. I propose, therefore, to use Dubois's word luciferin for the thermostable dialyzing substance of *Cypridina* in place of photophelein, and to use luciferase for the thermolable non-dialyzing substance in place of photogenin. The source of the photogenic substances can be designated by prefixing the name of the animal, as *Cypridina* luciferin, *Pholas* luciferin, etc. I suggest also that luciferin, when oxidized, be designated oxyluciferin.

Luciferin is found only in luminous animals. In non-luminous animals and probably also in luminous animals there is a second substance, which I have formerly included in the term photophelein (and which may be properly so called), that acts in a manner similar to the action of saponin, NaCl crystals, etc., upon the extract of *Cypridina* which has stood until the light disappears. When we allow a *Cypridina* extract containing luciferin and luciferase to stand, the luciferin is not completely oxidized, even though the extract is thoroughly aerated, but some of it is bound (adsorbed or combined?) by other substances in the extract. The saponin, NaCl crystals, and extracts of non-luminous animals act by setting free the bound luciferin, which is then oxidized and light appears. I suggest that the term photophelein be now applied to these substances in tissue extracts. They are not destroyed by boiling. On standing some are stable, while others are unstable.

The best way to rid a luciferase solution of the bound luciferin is to shake it thoroughly with chloroform. Such a solution will give no light with extracts of non-luminous animals or saponin, NaCl crystals, etc., but a brilliant light with *Cypridina* luciferin.

An insight into the *modus operandi* of saponin, NaCl crystals, or photophelein may be gained from the following experiment: Both luciferin and luciferase are adsorbed by many finely divided precipitates and colloidal particles, such as boneblack, $Fe(OH)_3$, kaolin, and others. If we take a colloidal $Fe(OH)_3$ solution of the proper concentration (which can only be determined by experiment), add some dilute luciferase to it, and then (after a minute) luciferin, no light will appear. This is because the luciferase has been completely adsorbed by the colloidal $Fe(OH)_3$, for if we now add some dilute luciferase to the above mixture, light will appear, but it will very quickly disappear, because the new luciferase added is again very rapidly adsorbed, but not so rapidly adsorbed that we fail to get light at first. On adding more luciferase we may again get a momentary light, but the additions can not be made indefinitely, because we finally reach a point where the colloidal $Fe(OH)_3$ has become saturated with luci-

ferase and then the mixture glows for a considerable time. It is obvious that for this experiment to succeed there must be more luciferin present than can be completely adsorbed by $Fe(OH)_3$ and so little luciferase present that it is completely adsorbed by the $Fe(OH)_3$. Suppose we have a mixture of $Fe(OH)_3$, luciferase, and luciferin complying with the above conditions. Can we in any way remove the luciferase from its adsorbed condition on the colloidal $Fe(OH)_3$? This might theoretically be done in two ways, and we actually find in practice that both methods are possible. Anything which precipitates the colloidal $Fe(OH)_3$ will decrease the surface available for adsorption of luciferase, and if the surface area is sufficiently decreased some luciferase may be forced into solution again, where it is able to oxidize the luciferin. If we add NaCl crystals to the colloidal $Fe(OH)_3$—dilute luciferase—more concentrated luciferin mixture, the $Fe(OH)_3$ is precipitated and light appears. If, in place of NaCl crystals, we add a trace of saponin, the colloidal $Fe(OH)_3$ is not precipitated, but light also appears. This is an example of the second method of removing luciferase from an adsorbed condition—namely, by using a material (saponin) which is more strongly adsorbed than the luciferase and which is able to replace it as adsorbed body. I believe these to be the explanations of the effect of NaCl crystals, saponin, etc., in giving light with luciferase solutions, except that the luciferase is in excess and a small amount of adsorbed (or combined) luciferin is present which is liberated by NaCl or saponin and gives light with luciferase. Photophelein probably acts in a manner analogous to the saponin.

I have considered the thermostable, dialyzing substance as similar to the luciferin of *Pholas*, despite the fact that Dubois finds *Pholas* luciferin destroyed at 70° C., whereas *Cypridina* luciferin is destroyed only by boiling for several minutes in an open beaker. I find that this destruction of *Cypridina* luciferin on short boiling is due to the increased rate of oxidation at the boiling-point and that no destruction of *Cypridina* luciferin will occur if boiled in an atmosphere of hydrogen.[1] *Cypridina* luciferin is truly thermostable, but is oxidized to oxyluciferin on boiling in the air. We may say that *Pholas* luciferin is *similar* but certainly not *identical* with *Cypridina* luciferin. If so, we should expect to obtain light on mixing *Pholas* luciferin and *Cypridina* luciferase, yet no light appears. Neither is there light on mixing *Cypridina* luciferin and *Pholas* luciferase, although the *Pholas* luciferase I was able to pre-

[1] I have endeavored to repeat this experiment with the luciferin of *Pholas* sent me by Professor Dubois, but without success. *Pholas* luciferin boiled in a current of hydrogen for 15 minutes would give no light when a crystal of $KMnO_4$ was added. The hydrogen was produced in a Kipp generator and may have contained a little air. In my experience short (20 to 40 seconds) boiling of *Pholas* luciferin does not completely destroy its power of producing light when a crystal of $KMnO_4$ is added.

pare from the material which Dubois sent me gave a rather faint light with *Pholas* luciferin.[1]

We have, therefore, at least three substances concerned in light production: luciferin, luciferase, and photophelein. Luciferin is a body oxidizing with light production, dialyzable, and relatively resistant to heat. Luciferase is destroyed by boiling, is non-dialyzable, and accelerates the oxidation of luciferin. While it may be used up in the reaction if mixed with a sufficient quantity of luciferin, luciferase has many of the characteristics of an enzyme and certainly as much right to be called an enzyme as the peroxidases of plants,which are also used up in the oxidation process. The *Cypridina* luciferase reaction appears to be specific to an extraordinary degree. Of many tried I have found no substances or plant or animal extracts which can take the place of luciferase[2] nor any substances[3] or plant or animal extracts[4] which can be oxidized with light production by luciferase. The light recorded with various extracts of luminous and non-luminous animals in my former paper is to be referred to the presence of photophelein, the third substance concerned in light production, which probably acts by assisting the luciferin-luciferase reaction in the manner already suggest d. Let us now turn to the oxidation product or products of luciferin.

When luciferin is oxidized it must be converted into some substance or substances, and I believe this change involves no fundamental destruction of the luciferin molecule, as it is a reversible process. I shall speak of the principal if not the only product formed as oxyluciferin. Most observers believe that a rather fundamental change occurs

[1] I believe the faint light obtained on mixing *Cypridina* luciferin and firefly or *Noctiluca* luciferase and *vice versa*, recorded in my former paper (*Am. J. Physiol.*, 1917, xlii, 328), where luciferin is called photophelein and luciferase is called photogenin, is not due to the oxidation of luciferin by luciferase of the second species, but is due to the presence of photophelein. I am led to this conclusion because the light is so faint, but can not be sure until the cases are reinvestigated. The mixing of luciferin and luciferase of different species or genera of luminous ostracods, especially if the color of their luminescence differed, would shed considerable light on this interesting question of specificity. A non-luminous Japanese species of *Cypridina* does not contain either luciferin or luciferase, but it does contain photophelein.

[2] I have tried the blood or extracts of many species of animals or plants, including those containing strong oxidizing enzymes both with and without H_2O_2 and have always failed to obtain light with *Cypridina* luciferin. Among others the juice of Indian pipe (*Monotropa*), potatoes and turnips (containing strong oxidases and peroxidases), the blood of the ox and a worm (*Arenicola*) (containing hemoglobin), the blood of the squid (*Loligo*), *Limulus*, and *Sycotopus* (containing hemocyanin), and extracts of *Chætopterus* (a luminous annelid) and the mollusc *Unio* (rich in manganese) were tried. Dubois reports that he has obtained light on mixing *Pholas* luciferin with the blood of divers molluscs and marine crustaceans (*Ann. Soc. Linn. de Lyons*, 1913). I can confirm this statement for an extract of *Unio*, but obtained no light with *Limulus* blood, *Sycotopus* blood, squid (*Loligo*) blood, or turnip or potato juice and *Pholas* luciferin. Evidently *Pholas* luciferin is much more readily oxidized with light production than *Cypridina* luciferin.

[3] The following oxidizable substances have been tested: æsculin, lophin, bergamot oil, pyrogallol, gallic acid, anilin, adrenalin, phenol, *a*-napthol, para-phenylen-diamine, ortol, orcin, hydrochinon, resorcin, pyrocatechin, tannin, benzidin, gum guaiac, amidol, *a*-napthylamine, and the chromogen of the false indigo plant (*Baptisia tinctoria*). Luciferase, with or without H_2O_2, will not accelerate the oxidative color change in any of the above compounds.

[4] I have regularly obtained a fair light on mixing luciferase well shaken with chloroform to set free any bound luciferin and boiled potato or turnip juice or boiled *Limulus* blood. The light is especially marked about the coagulum in the boiled *Limulus* blood. The significance of these results is not apparent.

when the photogenic substance is oxidized. Thus, the crystals of xanthin or some related substance in the reflecting layer of the firefly have been regarded as the oxidation products of the luminous material, thought to be nucleoprotein. Dubois[1] regards luciferin as a protein and states that it forms the same oxidation products as other proteins, amino-acids being mentioned as possible substances formed. It should be pointed out in this connection that the formation of amino-acids from proteins involves no oxidation, but an hydrolysis.

If we assume that the oxidation of luciferin changes the molecule but slightly, we at once think of comparing the change luciferin $\rightleftharpoons$ oxyluciferin with the change reduced hemoglobin $\rightleftharpoons$ oxyhemoglobin. The condition is, however, not so simple as this, for oxyhemoglobin will again give up its oxygen, providing the partial pressure of oxygen is sufficiently low, whereas oxyluciferin will not do this. We can not reduce oxyluciferin solution by exhausting the oxygen with an air pump.

There is another oxidation—reduction system which can also be easily reversed, but not by merely removing the oxygen—that is, the reduction of a dye such as methylene blue to its leuco-base. I believe the change which occurs when luciferin is oxidized is similar to that which occurs when the leuco-base of methylene blue or sodium indigo-sulphonate is oxidized to the blue dye.

My attempts to reduce the oxidation product of luciferin started from the observation that if one places a clear solution of luciferase in a tall test-tube, although it may give off no light at first when shaken, after standing a day or so a very bright light would appear on shaking. This was especially true when the luciferase had become turbid and ill-smelling from the growing of bacteria. Thinking that the bacteria produced a substance which could be oxidized by the luciferase, I tried growing bacteria and also yeast on appropriate culture media and after some days of growth mixing the culture media containing the products of bacterial or yeast growth with luciferase, expecting to obtain light; but no light appeared. However, if a little crude luciferase solution was added to the bacterial or yeast cultures and then allowed to stand for some hours, light appeared whenever they were shaken. Indeed, such cultures behaved much as a suspension of luminous bacteria which has used up all the oxygen in the culture fluid and will only luminesce when, by shaking, more oxygen dissolves in the culture medium. Realizing that in bacterial cultures in test-tubes anaerobic conditions soon appear, and also the strong reducing action of bacteria upon many substances (for instance, nitrates or methylene blue) under anaerobic conditions, it struck me that the bacteria might be utilizing the oxygen of the oxidation product of luciferin, reducing it to luciferin again. We must remember that since crude luciferase solution is a cold-water extract of a luminous animal allowed to stand until all the luciferin has

[1] Dubois, *Ann. Soc. Linn. de Lyons*, 1914, lxi, 169.

been oxidized, it must contain oxyluciferin as well as luciferase and will give light if the oxyluciferin is again reduced and oxygen admitted. This appears to be the correct explanation of the above experiments.

Not only bacteria but also tissue extracts have a strong reducing action in absence of oxygen. Thus, muscle tissue stained in methylene blue will very quickly decolorize (reduce) the methylene blue if oxygen (air) is kept away, but the blue color immediately returns if air is admitted. Oxyluciferin (*i. e.*, a solution of luciferin which has been completely oxidized by boiling or standing in air until it no longer gives light with luciferase), if mixed with a suspension of ground frog's muscle and kept in a well-filled and stoppered test-tube for some hours, is reduced to luciferin and gives a bright light if now poured into luciferase solution. Frog-muscle suspension alone or oxyluciferin alone give no light with luciferase, nor will a mixture of frog-muscle suspension and oxyluciferin, if shaken with air for several hours. Only if this last mixture be kept under anaerobic conditions is the oxyluciferin reduced.

The reducing action of tissues is said to be due to a reducing enzyme (reducase or reductase), itself composed of a perhydridase and some easily oxidized body such as an aldehyde.[1] In the presence of the perhydridase the oxygen of water oxidizes the aldehyde and the hydrogen set free reduces any easily reducible substance which may be present. There is a perhydridase in fresh milk, spoken of as Schardinger's enzyme,[2] which is destroyed by boiling. If some aldehyde is added fresh milk will reduce methylene blue to its leuco-base or nitrates to nitrites, upon standing a short time. If shaken with air the blue color returns. There is no reduction unless an aldehyde is added or unless some boiled extract of a tissue such as liver is added. The boiled-liver extract has no reducing action of its own, but supplies a substance similar to the aldehyde which has been spoken of as a co-enzyme. Milk will reduce methylene blue without aldehyde if bacteria are present in large numbers. Also, there is no reduction if the milk, methylene blue, and aldehyde are agitated with air. The temperature optimum is rather high, 60° to 70° C.

I find that milk is a favorable and convenient medium for the reduction of oxyluciferin and that it acts without the addition of an aldehyde or the presence of bacteria. There is probably a substance acting as the aldehyde in the luciferase-oxyluciferin solution. No light appears if milk is added to a luciferase-oxyluciferin solution, but if the mixture is allowed to stand in absence of oxygen light will appear when air is admitted. The air can be conveniently kept out by filling small test-tubes completely with the solution and closing them with rubber stoppers.

Oxyluciferin may also be readily reduced by the use of the blood of the horse-shoe crab (*Limulus*) allowed to stand until bacteria develop.[3]

[1] Bach, A., *Biochem. Z.*, 1911, xxxi, 443; xxxiii, 282; 1912, xxxviii, 154; 1913, lii, 412.
[2] Schardinger, F., *Chem. Zeit.*, 1904, xxviii, 704.
[3] Alsberg, C. L., *Journ. Biol. Chem.*, 1915, xxiii, 495.

This experiment is of special interest because the blood contains hemocyanin, which is colorless in the reduced condition and blue in the oxycondition. The color change thus serves as an indicator of the oxygen concentration in the blood. A sample of foul-smelling *Limulus* blood full of bacteria will become colorless on standing in a test-tube for 10 to 15 minutes, but the blue color quickly returns if shaken with air. Such a blood has the power of reducing oxyluciferin through the activity of the bacteria which it contains. Fresh blood has very little if any reducing action.

As almost all animal tissues contain reductases it is not surprising to find that a freshly prepared and filtered extract of *Cypridina* containing oxyluciferin and luciferase, which gives no light on shaking, will, on standing in a stoppered tube for 24 hours at room temperature, give light when air is admitted.[1] While this may be due to the development of bacteria with a reducing action, it does not seem likely, as under the same conditions methylene blue is not reduced in 24 hours and there is no turbidity or smell of decomposition in the tube. In 48 hours bacteria appear and methylene blue is also reduced. If we add chloroform, toluol, or thymol to the tubes of *Cypridina* extract to prevent the growth of bacteria, and allow them to stand 48 hours, upon admitting air the tube with chloroform gives no light, but the tubes with toluol and thymol do give light, although it is not so bright as if they were absent. I believe that these substances have a destructive action on the reductases, most complete in the case of chloroform.

I have not been able to demonstrate that a *Cypridina* extract will reduce methylene blue or nitrates to nitrites, either with or without the addition of acetaldehyde. This may be due to the fact that oxyluciferin, which is also present, may be reduced more readily than either nitrates or methylene blue, and so is reduced first.

Dubois[2] has described in *Pholas* a precursor of luciferin which he calls proluciferin, which is converted into luciferin by another enzyme, coluciferase. The proluciferin is not destroyed by boiling and the coluciferase will withstand a higher temperature than luciferase and may be freed of luciferase in this manner. He cites an experiment[3] to prove the existence of proluciferin and coluciferase in *Pholas*, but I have been unable to repeat this with *Cypridina*. One might suppose that on allowing an extract of *Cypridina* (luciferase) to stand in absence of oxygen some proluciferin, assuming this to be present, would be converted into luciferin, which would give light if air was admitted. But we can allow a boiled extract of *Cypridina* (containing no coluciferase) to stand with milk or muscle-tissue suspensions in absence of oxygen and upon admitting air and adding luciferase obtain light. As lucif-

[1] This experiment may also be performed with *Pholas* luciferase with a similar result.
[2] Dubois, *Compt. rend. Soc. Biol.*, 1907, 850; 1917, 964.
[3] Dubois, *Compt. rend. Soc. Biol.*, 1917, 964.

erase is found only in luminous animals it does not seem likely that a coluciferase would be widespread, but we do know that a reducing enzyme occurs in milk and tissue extracts—in fact is widespread. It seems more logical to interpret the above experiments as due to the reduction of an oxyluciferin to luciferin rather than the conversion of a proluciferin to luciferin.

Indeed, we can reduce oxyluciferin by means which do not involve the use of animal extracts and consequently are free from the objection that "coluciferase" may be responsible for the result, but which, nevertheless, are perfectly well-known reducing methods. Perhaps the best of these is reduction by palladium black and sodium hypophosphite. The latter is oxidized in presence of palladium and nascent hydrogen is set free.[1] The nascent hydrogen reduces any easily reducible substance which may be present, such as methylene blue or oxyluciferin. Oxyluciferin is not reduced by palladium alone or hypophosphite alone, but methylene blue is reduced by palladium black alone.

If hydrogen sulphide is passed through a solution of methylene blue the dye is very quickly reduced and becomes colorless. If the H_2S is driven off by boiling the colorless methylene-blue solution, the blue color again returns on cooling. Oxyluciferin can also be reduced to a certain extent by H_2S. Sulphur dioxide or oxides of nitrogen (prepared by the action of HNO_3 on Cu) had no reducing action on either methylene blue or oxyluciferin.

Dilute acid favors the reduction of oxyluciferin. If one saturates an oxyluciferin solution with CO_2 or adds a little dilute acetic acid and allows the solution to stand for 24 hours, a certain amount of reduction will occur. No reduction occurs if the solution is saturated with pure hydrogen and allowed to stand 24 hours. If one adds some Mg powder to oxyluciferin and then dilute acetic acid in successive additions as the acetic acid is used up in formation of Mg acetate, the oxyluciferin will be reduced relatively quickly. Nascent hydrogen is produced in the reaction and is no doubt the active reducing agent, while the acid accelerates the reduction. Soured milk also has quite a marked reducing action. Acid thus favors reduction and hinders oxidation, while alkali favors oxidation and hinders reduction of the oxyluciferin.

While I have not studied the properties of oxyluciferin as fully as those of luciferin, so far as I can judge both substances give the same general reactions and possess identical properties. If we make a concentrated hot-water extract of *Cypridina*, it will contain all the substances of the animal soluble in hot water and not coagulated by heat and may be spoken of as crude luciferin solution. If air is bubbled through this solution for some time, all the luciferin is oxidized and it may then be spoken of as crude oxyluciferin solution. Both crude luciferin and crude oxyluciferin solution are yellow in

[1] Bach, *Chem. Ber.*, 1909, xlii, 4463.

color, but I do not believe that either luciferin or oxyluciferin are yellow in color, because an ether or benzine extract of *Cypridina* is also yellow, although luciferase, luciferin, and oxyluciferin are all insoluble in ether and benzine. The yellow pigment which can be observed to make up part of the luminous gland of *Cypridina* is not luciferin or luciferase. It may be a pigment related to urochrome.

When tests are applied and precipitating reagents are added to crude luciferin and crude oxyluciferin solution they give identical results in each case. A complete account of the chemistry of luciferin will be found on pages 87 to 110, but a few of its more important properties are emphasized here.[1] If crude luciferin is saturated with $(NH_4)_2SO_4$ or $MgSO_4$ a flocculent precipitate forms which may be demonstrated to contain most of the luciferin (see page 93). Oxyluciferin solution also gives flocculent precipitates on saturation with $(NH_4)_2SO_4$ and $MgSO_4$ and these contain most of the oxyluciferin. To demonstrate this the precipitates, after washing, are dissolved in a small amount of water, mixed with fresh milk (or frog-muscle suspension) and allowed to stand in a stoppered tube for 24 hours. If any oxyluciferin is present it will be reduced to luciferin and give light when luciferase is added. One-half saturation with $(NH_4)_2SO_4$ or $MgSO_4$ or saturation with NaCl salts out no material from either crude luciferin or oxyluciferin solution. Picric acid gives no precipitate, but only an opalescence in both cases. In a similar manner it may be shown that most of the oxyluciferin is precipitated by phosphotungstic acid but not by acetic acid or CO_2, in this respect also agreeing with the behavior of luciferin. Like luciferin, the oxyluciferin will pass porcelain filters, dialyze through parchment or collodion membranes, is soluble in absolute alcohol, but not in ether or benzine, and is undigested by salivary diastase, pepsin, HCl, Merck's pancreatin in neutral solution, and erepsin. The salivary diastase and the pancreatin (containing amylopsin, trypsin, and lipase) were allowed to digest for 4 days at 38° C. without showing any evidence of digestive action. It is partially but not completely precipitated by basic lead acetate and by tannic acid.

As luciferin is so easily oxidizable a substance, we should expect to find that it will reduce just as glucose will reduce. However, a concentrated solution of luciferase has no reducing action on Fehling's (alkaline Cu), Barfoed's (acid Cu), Nylander's (alkaline Bi), or Knapp's (alkaline Hg) reagent. Glucose will reduce methylene blue in alkaline (not in neutral) solution, but luciferin will not reduce methylene blue in alkaline or neutral solution. It would seem, then, that luciferin must contain no aldehyde group. If so, we should expect to obtain reduction of some of the above reagents. Just what group is concerned in the oxidation is unknown at the present time, and in the absence of more experimental data speculation regarding it can be of little value.

[1] Dubois regards *Pholas* luciferin as a natural albumin and luciferase as an oxidizing enzyme made up of iron associated with a protein. "La Vie et la Lumière," Paris, 1914.

II. THE CHEMICAL NATURE OF CYPRIDINA LUCIFERIN AND CYPRIDINA LUCIFERASE.

PREPARATION OF MATERIAL.

The living animals are dried quickly in desiccators over $CaCl_2$ and may then be kept indefinitely in well-stoppered bottles containing a few lumps of $CaCl_2$ to remove any dampness in the air contained in the bottle. As the Cypridinæ dry, crystals of the salts of sea-water form upon them. These salts are hygroscopic and take up water, which results in a slow deterioration of the material unless precautions are taken to prevent access of water-vapor. Some of the salt and much of the débris of the animal's shell may be removed by a purely mechanical method. The whole Cypridinæ are finely ground in a mortar and the powder shaken with carbon tetrachloride. On standing, a layer of powder which contains most of the photogenic material, fragments of muscle, etc., is found floating at the surface of the CCl_4, while a layer of greater specific gravity at the bottom is found to contain very little photogenic material but much of the ground-up pieces of the shell. The CCl_4 extracts some of the fatty material and the remainder (with exception of lecithin) can be removed from the powdered animals by ether or petroleum ether in a Soxhlet extractor. These fat solvents do not dissolve or injure the photogenic substances in any way, although they are not sufficient for removal of all the lecithin from dried tissues. For this it is necessary to extract with hot alcohol, but as luciferin is soluble in absolute alcohol and more so in water-alcohol mixtures, we can not employ alcohol for this purpose. The lecithin in tissues which can not be extracted with ether is probably in combination with proteins. The treatment described above gives us a powder which lights brilliantly if moistened with water, both of the photogenic substances going into solution. It serves as the raw material for the isolation of luciferin and luciferase. A series of extractions of the dried material has also been made to determine the solubility of luciferin and luciferase in solvents other than water. Luciferase proved to be insoluble in all that were tried, but luciferin was soluble in many (see p. 109), although these non-aqueous solvents are less well adapted for its isolation than water. Accordingly both luciferin and luciferase have been extracted with pure water (distilled water or tap water) and purified by precipitation and re-solution.

From the dried, powdered, fat-free Cypridinæ an extract may be prepared with distilled water which contains luciferin, luciferase, all the proteins of the animal soluble in water, salts, and other water-soluble material. The extract, filtered through filter-paper, is yellow-colored and slightly opalescent and glows for some time. The light finally disappears, due to the oxidation of the luciferin. There is no change of

color on standing. If the extract solution be not too concentrated and is well shaken with air, all the luciferin will be oxidized and luciferase alone of the photogenic substances, together with oxyluciferin, will remain. Salts and other crystalloidal substances may be removed by dialysis, since luciferase does not dialyze. The solution is remarkably stable. I have allowed it to dialyze against running tap-water in a Schleicher and Schüll parchment diffusion thimble for two weeks and then against distilled water for one more week without any marked loss of luciferase. Indeed, solutions of luciferase may stand until they become foul and ill-smelling from bacterial decomposition without destruction of the luciferase. Solutions may be preserved free of bacterial development with toluol or chloroform for many months, but a slow destruction of the luciferase occurs; at the same time a precipitate forms. The luciferase is present in the colloidal state, as it does not pass collodion or parchment-paper membranes. A solution prepared in the above manner will be known as crude luciferase solution. For many purposes it is not necessary to previously remove the fat. Although the luciferase can be purified by repeated precipitation with a variety of substances, it loses strength during the process and the crude luciferase solution is the most powerful that can be obtained.

From the dried, powdered, fat-free Cypridinæ an extract may be prepared with *hot* water which contains the second of the photogenic substances, luciferin, all of the proteins of the animal not coagulated by heating, the remaining salts, and other material soluble in hot water. My procedure is to add boiling water directly to the dry powder, boil about 20 seconds, and filter quickly while hot. The filtrate is slightly opalescent, yellow-colored, and does not darken on standing. On cooling, this solution often glows faintly, but if heated to the boiling-point a second time the glow ceases and does not return on cooling, although its content of luciferin is diminished. This hot-water extract of Cypridinæ will be spoken of as crude luciferin solution. If the hot-water extract stands in a shallow dish at room temperature, the luciferin also disappears in the course of some hours, the time depending on its concentration. As already mentioned, this is due to oxidation of the luciferin apart from luciferase, as may be very easily determined by keeping the luciferin extract in absence of oxygen. I have kept such a solution in a test-tube covered with a layer of vaseline 1 inch in depth for 90 days, and at the end of that time it was capable of giving a brilliant light when mixed with luciferase. Luciferin and luciferase together in solution are likewise both stable in the absence of oxygen. I have kept such solutions in an evacuated tube or in a hydrogen atmosphere for many months and at the end of that time on admitting air a brilliant light appears.

The oxidative disappearance of luciferin, like other chemical reactions, is greatly accelerated at the higher temperatures. A solution of

luciferin which is boiled is very quickly oxidized and converted into oxyluciferin. Acid retards and alkali favors the oxidation of the luciferin (apart from luciferase). A solution of luciferin made slightly acid with HCl may be boiled for 25 minutes in the air without complete oxidation, whereas a neutral or slightly alkaline solution is quickly oxidized. A solution of luciferin will keep very well in a tall test-tube if the tube is left undisturbed. Diffusion of oxygen into the depths of the tube is very slow. Perhaps the best way to obtain a concentrated solution of luciferin is to filter the hot-water extract of *Cypridina* directly into a tall, narrow vessel and pass a current of CO_2 through it while cooling. The slight acidity and the anaerobic conditions both prevent oxidation. A little dilute acetic acid may be used in place of carbon dioxide. The oxidation product of luciferin, oxyluciferin, must also be present in crude luciferin solution to a greater or less extent, depending on the amount of oxidation which has taken place. Although both luciferin and oxyluciferin will pass collodion or parchment membranes, I have been unable to obtain them in crystalline form and presume that they also are present in the colloidal state.

With these preliminary remarks on the preparation of impure luciferase and luciferin solutions, let us examine the chemical and physical characteristics of the two substances. Two points must be borne in mind. *First*, the wonderful delicacy of the luciferin-luciferase reaction. One part of luciferase in about 1,700,000,000 parts water will give a visible light on the addition of luciferin, and *vice versa*. A precipitant must therefore precipitate absolutely; otherwise a mere trace of unprecipitated photogenic substance could be detected. It is also very necessary to make sure that in the vessel used in testing the precipitation none of the photogenic solution is adherent to the sides in such a position as to escape contact with the precipitant.

While a quantitative determination of the amount of luciferase (or luciferin) remaining in solution after adding a precipitant could be worked out by a method of dilution, *i. e.*, by determining how much water it would be necessary to add in order that the diluted solution would fail to give light with luciferin (or luciferase), this would be a tedious procedure. A rough estimate of the luciferin or luciferase in solution can be obtained by the brightness and duration of the luminescence, if one keeps in mind the following points: *First*, the greater the concentration of luciferin or luciferase the brighter the light. If luciferin is concentrated and luciferase is diluted the light will last a long time, while if luciferase is concentrated and luciferin weak a flash of light will occur which disappears very quickly. Bearing this in mind, one can judge fairly well whether precipitation has occurred and if it is only partial or very nearly complete.

Second, both luciferase and luciferin are adsorbed by many substances in a finely divided state—for instance, animal charcoal, freshly

precipitated $Fe(OH)_3$ or As_2S_3, infusorial earth, talc, or kaolin. If one mixes colloidal $Fe(OH)_3$ with dilute luciferase or dilute luciferin and then precipitates the $Fe(OH)_3$ with a little Na_2SO_4, the luciferase or luciferin will also be completely precipitated mixed with the $Fe(OH)_3$. If luciferin or luciferase are more concentrated there will be only partial precipitation with the $Fe(OH)_3$.

Most of the precipitants for luciferin and luciferase belong to the group of protein precipitants, but it must always be borne in mind that both of these substances may merely come down together with the proteins associated with them. It is, therefore, very difficult to say definitely from precipitation reactions whether luciferin and luciferase are proteins or merely adsorbed by protein precipitates. In many cases one can control this possibility by testing directly their adsorption on protein precipitates, and such experiments are referred to in the subsequent sections of this paper. Dubois[1] has come to the conclusion that *Pholas* luciferin and luciferase are proteins. He has been, however, by no means careful to give detailed description, or a critical analysis of his experiments, and he apparently has not considered the possibility of adsorption. Both *Cypridina* luciferin and luciferase certainly possess many properties in common with the proteins, but it will be more fitting to discuss their protein nature after describing in detail their chemical characteristics.

The following subjects will be considered: (1) Action of enzymes. (2) Salting out. (3) Alcohol and acetone. (4) Solubility in organic solvents. (5) Alkaloidal reagents. (6) Heavy metal salts. (7) Acids and alkalies. (8) Adsorbents.

ACTION OF ENZYMES.

Table 1 gives the results of enzyme experiments. A solution of crude luciferase (or crude luciferin) was mixed with the enzyme preparation and kept at 38° C. in an incubator for from 18 hours to 4 days. Controls were always employed, using previously boiled enzyme solution. Experiments were also made to determine if the particular enzyme preparation was active on its substrate and no experiments were considered in which this was found not to be the case. After the enzyme solution had acted for the proper length of time on the photogenic substances their light-giving power was tested by adding an equal amount of luciferin (or luciferase) to both control and active tubes, and comparing the brightness of the light resulting from the active tube with that of the control. In order to prevent oxidation the digests of luciferin were carried out in long test-tubes, full of solution, which were either corked or covered with a thick layer of vaseline. This procedure is not necessary in the case of luciferase.

[1] Dubois regards *Pholas* luciferin as a natural albumin and luciferase as an oxidizing enzyme made up of iron associated with a protein. "La Vie et la Lumière;" Paris, 1914.

The salivary diastase was a fresh-filtered saliva and the yeast in vertase a fresh-filtered extract of yeast ground with sand. All of the other preparations were made by dissolving the commercial enzyme powder in water. The erepsin was a solution of duodenal scrapings dried *in vacuo* and powdered, and the spleen, liver, and kidney substance was a solution of these glands dried quickly *in vacuo* and powdered. The three latter preparations probably contained proteolytic enzymes, although they did not digest fibrin under the same conditions with which they were tested with the photogenic substances. The erepsin in neutral solution did not digest fibrin to any extent, or albumen, but trytophane was produced from Witte's peptone.

Since both acid and alkali injure luciferase and alkali causes a very rapid oxidation of luciferin, some difficulty was experienced in working with pepsin, active only in acid, and with trypsin, most active in alkaline solution. As preparations containing trypsin were found to digest fibrin fairly rapidly in neutral solution, they were made up in water, and the experiments show that neutral trypsin solutions will digest luciferase. Presumably such neutral solutions would also digest luciferin if it were capable of digestion, but the results indicate that it is not. Pepsin could only be tested on luciferase with an amount of HCl lower than the optimum, otherwise the HCl alone is sufficient to destroy the luciferase. For this reason the action of pepsin was not so carefully investigated, but the result of the one experiment indicates that a slow digestion of luciferase occurs. Acid is not so destructive to luciferin, and 0.2 per cent HCl plus pepsin was found to possess no digestive power.

It will be noticed from table 1 (page 108) that of all the enzymes tried on luciferase only the proteolytic enzymes have any digestive power. Trypsin, erepsin, and pepsin HCl all have at least some digestive action. The commercial preparations of pancreas (pancreatin) usually contain some lipase (steapsin) and diastase (amylopsin), but as salivary diastase (ptyalin) and malt diastase did not digest luciferase and a sample of trypsin lacking lipase did digest luciferase, the destructive power of various "pancreatin" and "steapsin" preparations is unquestionably due to their trypsin content. That the destruction of luciferase is actually due to digestion and not to the injurious action of amino acids resulting from the digestion of proteins associated with the luciferase is shown by adding to luciferase the products of 4 days' (at 38° C.) tryptic digest of albumen (then boiled to destroy the trypsin) and keeping the mixture with toluol at 38° C. for 4 days more. The luciferase was found to be unaffected by the amino acids present.

These experiments all indicate that luciferase is a protein. As erepsin has a digestive action, one might suppose that it belongs to the group of proteoses, but too great reliance can not be placed on conclusions drawn from the action of erepsin, as this enzyme is said to

hydrolyze histones, protamines, casein, fibrin, and nucleic acid, in addition to proteoses and peptones.[1] Dubois finds that *Pholas* luciferase is digested by trypsin.[2]

On the other hand, none of the enzyme preparations tested had any action on luciferin. The proteolytic enzymes especially were studied with great care and with the result that no digestive action could be demonstrated. In one experiment not recorded in the table luciferin was mixed with a pancreatin preparation having active proteolytic power and kept at 38° for 4 days without digesting the luciferin. Erepsin also had no digestive action after 4 days at 38°. Merck's pancreatin (without toluol) had no digestive action after 8 days at 38° C. Dried, powdered cypridinæ were mixed with Merck's pancreatin and toluol and kept at 38° C. for 8 days. This digest was found to contain no luciferase but abundant luciferin. There is no doubt of the non-digestibility of the latter. As already mentioned, these digests must be carried out in absence of oxygen; otherwise the luciferin undergoes spontaneous oxidation and disappears, apart from any enzyme action. As we have seen, this oxidation involves no fundamental alteration of the luciferin molecule and the product (oxyluciferin) can be again reduced to luciferin. The oxyluciferin (like luciferin itself) is also undigested after 4 days' action of a pancreatin solution at 38° C. As all proteins except the racemized proteins and certain very insoluble albuminoids (elastin and keratin) are digested by trypsin, these experiments would seem to indicate that luciferin is not protein. However, this question will be again considered in the summary.

SALTING OUT.

If crude luciferase solution is saturated with crystals of NaCl at 20° C. no precipitate forms, but only a slight turbidity appears. The solution filters turbid and luciferase is found unharmed in the filtrate. Saturation with NaCl is a good method of preserving luciferase from the growth of molds and bacteria.

One-half saturation with $MgSO_4$ also produces a slight turbidity and luciferase is found unharmed in the filtrate. Complete saturation with $MgSO_4$ produces a fine precipitate, again dissolving in water which contains considerable luciferase. The opalescent filtrate also contains some luciferase, so that saturated $MgSO_4$ precipitates luciferase partially but not completely.

One-half saturation with $(NH_4)_2SO_4$ produces a precipitate which contains very little luciferase after washing with half-saturated $(NH_4)_2SO_4$. Most of the luciferase is found in the filtrate. On saturation with $(NH_4)_2SO_4$ an abdundant precipitate forms and *no* luciferase remains

[1] *Oppenheimer's Handb. d. Biochem.*, i, 554.
[2] Dubois, R., *Ann. Soc. Linn. de Lyons*, 1914, lxi, 161.

in the filtrate. The $(NH_4)_2SO_4$ precipitate dissolves completely in water and gives a brilliant light if mixed with luciferin.

If a crude solution of luciferin is saturated with NaCl or half-saturated with $MgSO_4$ or half-saturated with $(NH_4)_2SO_4$, no precipitate forms. The luciferin remains in solution. With saturated $MgSO_4$ the luciferin is partially precipitated; with saturated $(NH_4)_2SO_4$ it is almost completely precipitated, but a small amount of luciferin still remains in the filtrate. The addition of acetic acid to the point where precipitation of the crude luciferin solution is complete (probably a nucleoprotein or mucin is precipitated) and subsequent careful saturation with $MgSO_4$ or $(NH_4)_2SO_4$ do not completely precipitate the luciferin.

The above results can be confirmed by adding saturated solutions of NaCl or $MgSO_4$ or $(NH_4)_2SO_4$ to dry, powdered Cypridinæ. The powder glows strongly in saturated NaCl, which dissolves both luciferin and luciferase; weakly in saturated $MgSO_4$, which dissolves some luciferin but very little luciferase; and not at all in saturated $(NH_4)_2SO_4$, which dissolves no luciferase. On filtering the saturated $(NH_4)_2SO_4$ extract of dry Cypridinæ and pouring this into water no light appears, but if luciferase is now added a faint light appears, showing that a small amount of luciferin has gone into solution. These results are summarized in table 2 (page 109).

In order to determine if luciferin and luciferase are adsorbed on proteins or carbohydrates salted out of solution, the following experiments were performed:

Egg white was thoroughly mixed with about 3 volumes of dilute NaCl solution, a fairly dilute luciferase added, and then an equal volume of saturated $(NH_4)_2SO_4$ solution. The precipitate of "ovoglobulin" (ovomucin) which formed was allowed to stand for 10 minutes and then filtered off. The filtrate gave a good light with luciferin, whereas the mixture containing suspended precipitate gave a bright light. As luciferase is very slightly if at all precipitated from *Cypridina* extract with half-saturated $(NH_4)_2SO_4$, a certain amount must have been adsorbed on the ovomucin precipitate.

A similar experiment with egg white containing luciferase salted out by saturation with NaCl indicated that some luciferase was adsorbed by the fine precipitate of ovomucin salted out with NaCl.

A neutral solution of sodium caseinogenate is completely salted out by half-saturation with $(NH_4)_2SO_4$. If luciferase is also present most of it but not all goes down with the caseinogen precipitate. The precipitate redissolves in water and the luciferase again becomes active.

Gelatin solution (1 per cent) containing luciferase is also completely precipitated with $(NH_4)_2SO_4$ and the gelatin precipitate carries down most of the luciferase. The gelatin precipitate containing adsorbed

luciferase does not redissolve readily in water, but gives a bright light on mixing with luciferin, so that the adsorbed luciferase must again go into solution.

Starch paste (with the grains well broken up by 15 minutes in the autoclave at 117° C.) containing luciferase is completely coagulated by half-saturation with $(NH_4)_2SO_4$, but the starch carries down very little of the luciferase, although a small amount is adsorbed. Very little of the starch again goes into solution in cold water. Corn-starch grains suspended in water also adsorb very little luciferase as compared with inorganic precipitates of various kinds.

Luciferin is only slightly adsorbed by starch coagulated with an equal volume of $(NH_4)_2SO_4$, and by ovomucin precipitated by saturation with NaCl or half-saturation with $(NH_4)_2SO_4$, but a considerable amount is taken up by Na caseinogenate precipitated with an equal volume of $(NH_4)_2SO_4$ and 1 per cent gelatin precipitated with an equal volume of $(NH_4)_2SO_4$. The light is especially bright about the particles of gelatin containing adsorbed luciferin when luciferase is added.

Since the amount of adsorption depends not only on the adsorbent but upon its surface area, it is impossible to judge of the relative amounts of luciferin and luciferase adsorbed by different proteins or carbohydrates in the above rough experiments. However, care was taken to see that in all experiments the protein precipitated was present in far greater proportion than the protein associated with the photogenic substances in crude luciferase or crude luciferin solutions—*i. e.*, the conditions for adsorption were purposely selected to favor adsorption. The results indicate that adsorption does occur. It is, however, not complete even under these favorable conditions, so that we must conclude that the results obtained by salting out crude luciferin and luciferase solutions give us a fairly accurate clue as to the properties of these substances were they alone in pure solution. Where we find luciferase completely precipitated from crude solution, as by saturation with $(NH_4)_2SO_4$, we may be sure that the luciferase is actually precipitated and is not merely completely carried down as an adsorption complex. But where we find only partial precipitation, as by saturation with $MgSO_4$, it is difficult to say how much precipitation would occur were other proteins absent, although we can be sure that the precipitation would not be complete.

Although we ordinarily think of the proteins as the substances, *par excellence*, capable of being salted out of solution, the property is characteristic of many emulsion colloids, notably soaps, polysaccharides, and phospholipins. However, neither luciferase nor luciferin are soaps, because they are not precipitated by $CaCl_2$, nor phospholipins or galactolipins, because both are insoluble in ether and benzene, hot or cold. It is possible that they are of polysaccharide nature, as starch and

glycogen are nearly if not completely precipitated by saturating their solution with $(NH_4)_2SO_4$. But the polysaccharides are not precipitated by the phosphotungstic acid, whereas luciferase is completely precipitated and luciferin very nearly completely precipitated (see p. 98).

The evidence from salting-out experiments indicates that both luciferin and luciferase are proteins, the former on the border-line between proteoses and peptones, the latter a more complicated protein but not a globulin. Dubois[1] finds *Pholas* luciferin completely precipitated by saturation with $(NH_4)_2SO_4$, but not by $MgSO_4$ or NaCl.

ALCOHOL AND ACETONE.

If strong ethyl alcohol or acetone is added to a solution of crude luciferase an abundant precipitate forms. This precipitate is found to contain the luciferase, which is separated completely from solution by alcohol between 50 and 60 per cent and by acetone between 70 and 80 per cent. We are dealing in both cases with a precipitation and not a coagulation of luciferase. The precipitates partially redissolve in water, and if the solution is filtered luciferase is found in the filtrate. Indeed, the precipitates from addition of alcohol or acetone to crude luciferase may stand under alcohol (95 per cent) or acetone (90 per cent) respectively for 35 days without complete destruction of the luciferase, but there is always a diminution of activity which is no doubt eventually complete. The alcohol or acetone precipitate may be washed and dried and will give light whenever luciferin is added.

If strong ethyl alcohol or acetone is added to a solution of crude luciferin a precipitate also forms, but it is not nearly so voluminous, since the heat-coagulable proteins are absent from the luciferin solution. With alcohol the precipitate is very fine; with acetone it is flocculent and clumps together readily.

The precipitates washed once with 95 per cent alcohol and acetone respectively dissolve completely in water, but these solutions give only a faint or no light if luciferase is added. On the other hand, the filtered alcohol and acetone solutions contain considerable amounts of luciferin. The absence of luciferin in the alcohol and acetone precipitates is only apparent. It is really partly precipitated by alcohol and acetone, but is largely oxidized during the process of testing. Experiments indicate that oxyluciferin is present in the precipitates and can be reconverted into luciferin by appropriate methods. These experiments indicate that alcohol and acetone only partially precipitate luciferin, but completely precipitate luciferase.

They are confirmed by extracting the dried, powdered Cypridinæ with alcohol or acetone solutions of various strengths. Luciferin is soluble in all strengths, even in absolute alcohol and acetone, although it is sparingly soluble in the latter. Luciferase is only sparingly soluble

[1] Dubois, *Ann. Soc. Linn. de Lyons*, 1914, lxi, 161.

in strengths of alcohol containing over 40 per cent water and in acetone containing over 30 per cent water. In weaker alcohol and acetone solutions it is more soluble. Indeed, the dry *Cypridina* powder will glow faintly in 70 per cent acetone and 50 per cent alcohol, but not in 90 per cent acetone or 70 per cent alcohol. In this case we have the two substances, luciferin and luciferase, in intimate contact in a concentrated condition. If they are present in less concentration, as in a glowing mixture of the two in water, the addition of alcohol or acetone to about 20 per cent is sufficient to extinguish the light.

Boiling absolute alcohol will extract a considerable amount of luciferin, and such a solution is quite stable even if exposed to air, providing the alcohol is not allowed to evaporate away. In alcohol-water and acetone-water mixtures the luciferin oxidizes rather readily and disappears in the course of a day or so. I am inclined to think that alcohol may also have a destructive action on luciferin, because an absolute-alcohol extract of luciferin sealed in a glass tube in absence of air showed the presence of only a very small amount of luciferin when tested after a period of 90 days. A very small amount of a fine precipitate was present.

SOLUBILITY IN ORGANIC SOLVENTS.

The solubility of luciferin and luciferase in a number of pure organic solvents was tested by extracting the dried, powdered Cypridinæ, filtering, and then testing the filtrate by adding luciferase and luciferin, respectively. Small pieces of the photogenic gland will sometimes pass through ordinary filter-paper, but can always be recognized as isolated, brightly luminous dots appearing when the solvent is tested for photogenic material. A heavy blotting-paper holds back these fine particles and was used for filtering, except in the case of very viscous solvents. The results are summarized in table 2.

It will be noticed that luciferase is not extracted by any of the solvents tried, whereas luciferin is soluble in a considerable number of them. Methyl alcohol, ethyl alcohol, propyl alcohol, benzyl alcohol, ethyl acetate, glycerine, and glycol all dissolve a considerable amount of luciferin. Solvents non-miscible with water, such as benzyl alcohol or ethyl acetate, give up their content of luciferin to the water phase and it then luminesces if luciferase is present. In the case of the homologous series of aliphatic alcohols, the higher the alcohol in the series the less luciferin will it dissolve. The same is true for the homologous series of esters.

Luciferin is fairly stable in methyl alcohol, ethyl alcohol, propyl alcohol, benzyl alcohol, and glycerine if no water is present, but rather quickly disappears in acetone, glycol, and ethyl acetate, presumably because oxidation occurs more rapidly in the latter solvents. In the

typical fat solvents, as ether, chloroform, benzol, etc., the luciferin is insoluble. Luciferin is also soluble in glacial acetic acid but not in anilin.

There is nothing in the solubility relations of luciferase to indicate that it is not a protein. On the other hand, the solubilities of luciferin are certainly unusual for a natural protein. The best-known class of proteins soluble in alcohol are the prolamines of plants; but the prolamines are insoluble in water and absolute alcohol. Zein, the prolamine of corn, is soluble in 90 per cent ethyl alcohol, methyl and propyl alcohols, glycerine heated to 150° C., and glacial acetic acid.[1] Recently Osborn and Wakeman[2] have described a protein from milk having solubilities similar to those of gliadin, the prolamine of wheat. Welker[3] has described a substance (obtained from Witte's peptone, giving the biuret, Millon, and Hopkins-Cole tests) which is soluble in water and absolute alcohol, but not in ether, and it is quite possible that others of the peptones are soluble in absolute alcohol. On the other hand, some proteins in the absence of salts form colloidal solutions in strong alcohol from which they may be precipitated by an appropriate salt.[4] As the absolute alcohol extract of Cypridinæ was made from dry material containing the salts of sea-water, some salt was present, but there is always the possibility of sol formation.

If we extract dried Cypridinæ which have previously been thoroughly extracted with benzine, with 800 c.c. boiling absolute alcohol for an hour, filter the alcohol extract through blotting-paper and hardened filter-paper, quickly evaporate the filtrate to dryness on the water-bath, and dissolve the residue in a small quantity of water saturated with CO_2, we obtain a yellow opalescent solution which gives a bright light with luciferase. This solution contains some protein or protein derivatives, as it gives a very faint Millon reaction, a good positive ninhydrin test, reddish blue in color, but no biuret reaction. It precipitates with tannic and phosphotungstic acids, but not with picric, acetic, trichloracetic, or chromic acids or on saturation with $(NH_4)_2SO_4$. The phosphotungstic precipitate is not due to the presence of calcium. The extract gives a faint Molish reaction for carbohydrates. As the evidence points to the presence of some protein material in the absolute-alcohol extract of Cypridinæ, it is possible that this protein is luciferin. It should be emphasized, however, that the Millon reaction was very faint, although the ninhydrin was quite marked.

ALKALOIDAL REAGENTS.

Phosphotungstic acid, tannic acid, and picric acid were selected from among the alkaloidal reagents to study precipitating power.

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[1] Osborn, T. B., *Ergeb. Physiol.*, 1910, x, 47.
[2] Osborn, T. B., and A. J. Wakeman, *J. Biol. Chem.*, 1918, xxxiii, 243.
[3] Welker, W. H., *Biochem. Bull.*, 1912, ii, 70.
[4] Private communication from T. B. Osborn.

A solution of phosphotungstic acid added to crude luciferase solution gives a voluminous precipitate which, if washed quickly in running water on the filter and then suspended in water, does not completely dissolve, but gives a good light if luciferin is added. It dissolves if a trace of dilute NH_4OH solution is added. The filtrate gives no light with excess luciferin, even though it contains some material which will precipitate on addition of more phosphotungstic acid.

Tannic acid added in slight excess to luciferase gives an abundant precipitate which contains luciferase and will give a good light with luciferin if suspended in water. It does not completely dissolve, even on addition of dilute NH_4OH solution. The filtrate gives a very faint light on addition of an excess of luciferin, even though an excess of tannic acid was used to precipitate and all precipitable material was thrown down.

Saturated aqueous picric acid added in equal volume to crude luciferase solution gives a not very abundant precipitate which, if filtered off, washed quickly with water, and suspended in water, does not completely dissolve. If luciferin is added the precipitate does dissolve and a good light appears. Dilute NH_4OH solution causes the precipitate to dissolve. The filtrate gives a very faint light when an excess of luciferin is added, even though everything was precipitated that would precipitate with picric acid and an excess of the latter was present. The excess of picric acid was not sufficient to harm the luciferin used in testing.

Attempts were made to study the adsorption of luciferase by picric acid, tannic acid, and phosphotungstic acid precipitates of egg albumen. The difficulty is that these precipitates tend to go in solution again and it is very difficult to remove by washing (centrifuging and decantation) the precipitant. This is especially well observed in the case of the picric acid-egg albumen precipitate, where the supernatant liquid has a yellow color after prolonged washing. However, the results show that very little adsorption takes place, not nearly enough to explain the complete or nearly complete precipitation of luciferase from crude solution by these reagents.

Luciferase is, therefore, completely precipitated by phosphotungstic acid and very nearly completely precipitated by tannic and picric acids without harm.

Phosphotungstic acid added to a solution of crude luciferin to slight excess gives a bulky precipitate which can be filtered off and the filtrate is perfectly clear. It gives no further precipitate with phosphotungstic acid, but contains a small amount of luciferin, as a faint light appears when luciferase in *excess* (since luciferase is also precipitated by the excess phosphotungstic acid present) is added. The precipitate washed on the filter with dilute phosphotungstic acid does not dissolve completely in water, but partly dissolves, forming an opalescent solution. It is brought into solution by adding crude luciferase or a trace of dilute

NH_4OH solution. The precipitate contains luciferin and gives a brilliant light with luciferase. It is probably the slight alkalinity of the luciferase solution which causes re-solution of the luciferin precipitate. The addition of a small amount of HCl to the phosphotungstic-luciferin filtrate causes further precipitation, leaving a clear supernatant fluid containing no trace of luciferin.

Tannic acid likewise gives a voluminous precipitate with luciferin solutions and the filtrate is clear at first but may become cloudy in a short time, especially at the surface, unless considerable tannic acid is added. If an excess of tannic acid is present, as indicated by absence of further precipitate on adding more tannic acid, a small amount of luciferin is present in the filtrate, but practically all is precipitated. The precipitate does not completely dissolve in water, even if NH_4OH is added, but gives a bright light when luciferase is added. The addition of a small amount of acetic acid + NaCl or HCl does not cause further precipitation. Both the phosphotungstic and tannic acid precipitates can be dried and will again give light with luciferase if redissolved.

Picric acid added to luciferin solution gives no good precipitate, but only an opalescence or turbidity. This is true even when picric acid is present in considerable excess, as on addition of an equal volume of saturated aqueous picric acid. A little dilute acetic acid or HCl does not cause precipitation. The solution filters turbid and luciferin may be demonstrated in the filtrate on adding luciferase. A yellow light due to the picric acid in solution appears. A little dilute NH_4OH clears the turbidity.

If we add acetic acid to luciferin solution until the precipitation (probably a precipitate of mucin) is complete and then dilute $K_4Fe(CN)_6$ solution, no further precipitates form and luciferin is found abundantly in the filtrate, which gives no further precipitate with $K_4Fe(CN)_6$ and acetic acid. The results are recorded in table 3 (p. 110).

Luciferin is, therefore, very nearly completely precipitated by phosphotungstic and tannic acids, but not by picric acid. It is completely precipitated unharmed by phosphotungstic acid + HCl, but not by tannic acid + HCl or picric acid + HCl. It is not precipitated by potassium ferrocyanide and acetic acid. Dubois[1] finds *Pholas* luciferin to be completely precipitated by picric acid.

HEAVY METAL SALTS.

Some of the heavy metal salts (Pb acetate, $AgNO_3$, $CuSO_4$) precipitate in sea-water. As both crude luciferin and luciferase solutions, although prepared with distilled water, contain small amounts of the salts of sea-water, a slight precipitate would be formed upon addition of lead acetate. It is too small in amount to interfere with the action

[1] Dubois, *Ann. Soc. Linn. de Lyons*, 1913.

of lead acetate as a precipitant, but it must be borne in mind that some of the luciferin or luciferase might be adsorbed on any precipitate formed, as this is especially likely to occur with inorganic precipitates. Another difficulty encountered in precipitating protein solutions with heavy metal salts is that in excess of heavy metal salt some of the precipitate may redissolve. However, certain definite results were obtained by precipitating crude luciferin and luciferase with lead and mercury salts (the usual protein precipitants), and these are described below, although their interpretation may be open to question.

Neutral lead-acetate solution was added to crude luciferase solution, drop by drop, until no further precipitate formed. The filtrate was clear and gave no further precipitate on adding more lead acetate. It gave a good light with excess of luciferin. The precipitate suspended in water does not dissolve, but upon addition of luciferin gives a bright light.

Basic lead acetate added drop by drop gives a bulky precipitate, leaving a clear filtrate which gives no further precipitate on addition of more basic lead acetate. This filtrate gives no light with excess luciferin, but the precipitate washed once with water on the filter gives a bright light.

Saturated $HgCl_2$ solution was added drop by drop to luciferase solution until no further precipitate occurs. The filtrate is perfectly clear and gives a bright light with luciferin, although it gives no further precipitate with $HgCl_2$. The precipitate washed on the filter for 20 hours in running water is suspended in water. It does not completely dissolve, but on addition of luciferin gives a fair light.

Luciferase, therefore, is completely precipitated by basic lead acetate, nearly completely precipitated by neutral lead acetate, but not precipitated by mercuric chloride.

If a saturated solution of $HgCl_2$ is added drop by drop to luciferin solution a precipitate forms. If this is filtered off, washed with dilute $HgCl_2$, and suspended in water, it does not completely dissolve, but gives a good light if an excess of luciferase is added. The opalescent filtrate which gives no further precipitate with $HgCl_2$ gave a fair light on adding an excess of luciferase. Mercuric chloride and acetic acid almost completely precipitate luciferin from solution.

Neutral lead-acetate solution added to luciferin gives a heavy precipitate. The solution filters cloudy at first, even though no further precipitate forms on adding lead acetate, but soon filters clear. This clear filtrate containing some Pb gives a bright light on addition of excess of luciferase. The precipitate was washed several times on the filter and by decantation with dilute Pb acetate, and suspended in water. It does not completely dissolve, but gives a good light with luciferase. Lead acetate and acetic acid give no good precipitate with crude luciferin.

Basic lead acetate gives a voluminous heavy precipitate with crude luciferin. After heating, the solution was filtered and the clear filtrate, which gave no further precipitate with basic lead acetate, gave a brilliant light with luciferase. The precipitate washed twice with water on the filter does not dissolve in water, but suspended in water gives a faint light on adding luciferase. Uranium nitrate + acetic acid does not completely precipitate luciferin from solution. These results are recorded in table 3.

Luciferin is therefore not completely precipitated from solution by mercuric chloride with or without acetic acid, neutral lead acetate, or basic lead acetate, or uranium nitrate and acetic acid.

ACIDS AND ALKALIES.

As alkalies (KOH or NaOH in small, NH_4OH in greater concentration) precipitate the Mg of sea-water, this salt should be removed from a crude solution of luciferin and luciferase before studying the precipitating effects of these substances. This can be done by the addition of a small amount of sodium pyrophosphate, which forms Ca and Mg pyrophosphates, insoluble in water. The precipitates are removed by filtration and the photogenic substances are found unharmed by the addition of pyrophosphate.

Neither luciferin nor luciferase are precipitated by addition of dilute NaOH or dilute NH_4OH to their crude solutions, first rendered free of Ca and Mg.

Dilute acetic acid added to luciferase solution gives a fine precipitate which is filtered off and washed with running water for 24 hours. It does not dissolve completely in water and gives only a faint light with luciferin. The precipitate is probably a mucin and carries down some adsorbed luciferase. The filtrate is perfectly clear, gives no further precipitate (sometimes a slight cloudiness on standing) with acetic acid, but a bright light with luciferin. The addition of slightly more acetic acid results in a clear filtrate giving no light with excess luciferin. The precipitate on the filter does give a faint light with excess luciferin, but appears to be injured by the acid.

Saturation with CO_2 causes a precipitation in crude luciferase solution, but the filtrate gives a brilliant light with luciferin.

Dilute acetic acid added to concentrated luciferin gives a stringy precipitate. If filtered off, the filtrate is slightly opalescent, but does not become more cloudy or precipitate if more acetic acid is added. It gives a bright light if luciferase is added to it. The precipitate does not completely dissolve in water, but if washed with water and suspended in water gives a fair light with luciferase. This precipitate is probably a mucin containing some adsorbed luciferin. In the luminous gland of *Cypridina* there is a material which stains as does mucin, and it would

be found in the hot-water extract of the whole animal. The acetic-acid precipitate does not form readily if the luciferin solution is hot when acetic acid is added. A little dilute NH_4OH will dissolve the precipitate and it can be reprecipitated by acetic acid. The filtrate from the acetic-acid precipitation gives a voluminous precipitate with phosphotungstic acid, which does not carry down all the luciferin unless a little HCl is also added, when luciferin is completely precipitated and may be demonstrated in the precipitate.

Dilute HCl alone gives a precipitate with crude luciferin solution, but is almost completely dissolved in an excess of dilute HCl.

Saturation of a solution of crude luciferin in presence of some NaCl with carbon dioxide does not cause precipitation nor an increase in turbidity.

Dilute trichloracetic acid gives a stringy precipitate with crude luciferin solution similar to that with acetic acid, but abundant luciferin is found in the clear filtrate, which gives no further precipitate with trichloracetic acid. These results are recorded in table 3.

Hence neither luciferin nor luciferase is precipitated from crude solution by dilute NH_4OH or NaOH or by dilute acetic acid, and neither of them can belong to the group of histones (precipitated by dilute NH_4OH), or mucins or nucleoproteins, or such phosphoproteins as caseinogen, which are precipitated by dilute acetic acid. A mucin or nucleoprotein is precipitated from both crude luciferase and luciferin solution by dilute acid and carries down *some* luciferase and *some* luciferin in the adsorbed state. It is easy to demonstrate that such an adsorption might occur. A solution of sodium caseinogenate mixed with either luciferin or luciferase and precipitated with acetic acid will carry down a considerable amount but not all of the luciferin or luciferase. Luciferin is not, but luciferase is, injured by an excess of dilute acetic acid.

Dubois[1] reports that *Pholas* luciferin is not precipitated by carbonic acid in neutral solutions or by acetic acid, except in presence of neutral salts, and that it forms an insoluble alkali albuminate with NH_4OH. The latter is possibly a $Mg(OH)_2$ formed from the magnesium in the luciferin solution.

ADSORBENTS.

Proteins are usually separated from their solutions by one or another of the following methods: (1) Heat coagulation (in trace of acid). (2) Precipitation by alcohol or acetone (in large excess). (3) Precipitation by heavy metal salts (basic lead acetate, $HgCl_2$ and acid, uranium acetate and acid, etc.). (4) Alkaloidal reagents (phosphotungstic, tannic, picric acids, etc.). (5) Salting out (by $MgSO_4$ and acid,

[1] Dubois, *Ann. Soc. Linn. de Lyons*, 1914, lxi, 161.

$(NH_4)_2SO_4$, etc.). (6) Digestion by proteolytic enzymes. (7) Adsorption (by chloroform, toluol, $Fe(OH)_3$, kaolin, and gum mastic).

We have already noted the behavior of luciferase and luciferin toward the first six methods. Both of these substances can also be separated from solution by adsorption on appropriate material—in fact, they are rather readily adsorbed, especially by inorganic precipitates. Their complete adsorption is usually merely a matter of obtaining sufficient adsorbing surface area. For this reason comparative studies on adsorption of different materials are difficult to carry out, because we can not be sure of uniform surface area. However, it may be of interest to record a few of my experiments on adsorption.

A neutral dilute solution of luciferase was found to be completely adsorbed by boneblack, $Fe(OH)_3$, As_2S_3, infusorial earth (SiO_2), talc, and kaolin; nearly completely adsorbed by asbestos, pumice, $CaCO_3$, and $MgCO_3$; not nearly completely adsorbed by ground glass, sulphur powder, gelatin or agar-agar threads, heat-coagulated egg albumen, fresh precipitated caseinogen, cotton, or corn starch.

A solution of luciferase shaken with five successive additions of fresh chloroform, until the chloroform is no longer emulsified but separates as readily as with water, is reduced considerably in luciferase content, but the luciferase is not completely removed by the chloroform.

Neutral luciferin is completely adsorbed by boneblack, $Fe(OH)_3$, kaolin, talc, and $CaCO_3$, but not by many organic precipitates, as caseinogen, corn starch, or gelatin threads. There is the difficulty in studying adsorption of luciferin that oxidation may be accelerated by the presence of finely divided material.

Luciferin can also be removed practically completely from solution by gum mastic according to the method of Michaelis and Rona[1] for removing proteins from blood serum.

CONCLUSIONS.

There seems to be very little doubt that luciferase is a protein or so closely associated with proteins that their removal destroys its characteristic properties. Thus, it is destroyed by proteolytic enzymes in neutral solution, completely precipitated by saturation with $(NH_4)_2SO_4$, almost completely precipitated by $MgSO_4$ but not by NaCl or half-saturated $(NH_4)_2SO_4$, insoluble in all except aqueous solvents, readily absorbed by various inorganic precipitants, completely precipitated by phosphotungstic acid and basic lead acetate, and almost completely precipitated by tannic and picric acids. The particular group of proteins to which it belongs may be arrived at by a process of exclusion. Some of the above characteristics, together with the fact that it will not dialyze and is coagulated on heating, are sufficient to exclude it

[1] Michaelis, L., and P. Rona, *Biochem. Z.*, 1907, ii, 219; iii, 109; iv, 11.

from the class of hydrolyzed derived proteins (proteoses, peptones, and peptids). Of the seven groups of simple proteins, it can not belong to the globulins—because it is not precipitated on dilution with water or on half-saturation with $(NH_4)_2SO_4$—or glutenins (because soluble in water), or prolamines (because insoluble in 70 per cent alcohol), or albuminoids (because of its solubility in aqueous solvents), or histones (because not precipitated by dilute NH_4OH), or protamines (because coagulated on heating). There remain only the albumins and the groups of conjugated proteins—the glycoproteins (mucins and mucoids). nucleoproteins, phosphoproteins (caseins), lecithoproteins (vitellins), and chromoproteins. It is not a chromoprotein (because not colored), or a vitellin-like protein which has properties similar to the globulins, or a nucleoprotein, casein-like or mucin-like protein, because not precipitated by dilute acetic acid and because coagulated on boiling. It is not a mucoid (similar to the mucins but not precipitated by acetic acid) because coagulated on boiling. The latter contains a carbohydrate group, but I can not state definitely at the present time whether pure luciferase contains a carbohydrate group or not. There remains only the group of albumins, with which the properties of luciferase agree completely.

Dubois believes *Pholas* luciferase to be an oxidizing enzyme similar to the oxydones of Batelli and Stern,[1] because it is readily destroyed by fat solvents, such as chloroform, strong alcohol, etc. He has detected iron in a luciferase solution which has dialyzed against running water for a long time and believes it to be made up of protein in combination with iron and to act as an "oxyzymase ferrique."[2] *Cypridina* luciferase, on the other hand, is not *readily* destroyed by fat solvents. Toluol and chloroform are good preservatives and I often make use of them for this purpose, keeping the luciferase solutions for many months. Professor A. H. Phillips, of Princeton University, has very kindly analyzed some whole, dried Cypridinæ for me and finds iron, copper, and manganese, but no zinc or vanadium present. Whether these metals are connected with the action of *Cypridina* luciferase is uncertain, but it is a significant fact that all three metals concerned in oxidations occur in the animal.

Although a large amount of luciferin mixed with a small amount of luciferase will use up all of the latter,[3] I agree with Dubois that lucif-

[1] Batelli, A., and Stern, *Biochem. Z.*, 1914, lxvii, 443.

[2] Dubois, R., *Ann. Soc. Linn. de Lyons*, 1914, lxi, 161.

[3] If one mixes concentrated luciferin and weak luciferase the light which appears will last a long time before going out. After the light disappears, if one dilutes this mixture with water or adds more luciferin no further luminescence occurs, but if one adds more weak luciferase, light again is produced and lasts a considerable time. The fact that no more light appears on diluting the concentrated luciferin-weak luciferase mixture with water shows that the enzyme has not been inhibited by reaction products. If so, the dilution of these reaction products should allow the system to proceed to a new (false) equilibrium with production of light. Dubois (*Ann. Soc. Lynn. de Lyon*, xlix, 105, 1917) has misunderstood my statement fn regard to this.

erase has sufficient properties in common with the enzymes as a class to be considered an enzyme. The peroxidases are well known to be used up in the reactions they accelerate. All workers on enzymes agree that the more they are purified the less active they become. The chemical procedures necessary to remove foreign material bring about irreversible changes in the enzyme itself, a characteristic also of many protein groups and of the colloidal state in general. This is true in the case of luciferase, for the crude luciferase solution is the most active preparation that can be obtained.

I believe that *Cypridina* luciferase should be placed in a class of oxidizing enzymes by itself—a group having the chemical reactions of an albumin, possibly in combination with some heavy metal, and which, so far as we know, acts specifically on only one substance, *Cypridina* luciferin. It resembles the plant peroxidases in resisting the action of chloroform, toluol, etc., but will not oxidize any of the hydroxy-phenol or amino-phenol compounds[1] so readily oxidized by the peroxidases, nor will the peroxidases oxidize luciferin with light production. Dubois's researches show that *Pholas* luciferase differs in some properties from *Cypridina* luciferase and my own work[2] indicates that firefly luciferase is more like that of *Pholas*. A comparative study of other species of luminous animals is most needed in order to delimit more accurately the class of luciferases as a whole.

Luciferin presents many characteristics in common with the proteins, but two, which, to say the least, throw doubt on its protein nature. I refer to its peculiar solubility (in alcohols, esters, and glacial acetic acid), and its resistance to digestion by proteases, even by trypsin, which has almost universal digestive action. These two peculiarities have already been discussed in the body of the paper and there is no need of repeating the discussion here. We can only say that if a protein, luciferin must belong to a new group differing from *known natural* proteins in these respects. In general characteristics this new group would fall somewhere on the border-line between the proteoses and peptones. Perhaps it would not be surprising to find in nature proteoses or peptones soluble in absolute alcohol. We know also that some NH-CO linkages of proteins are broken down with great difficulty

[1] Because of the ease with which many of these hydroxyphenyl compounds undergo autoxidation one must always compare the color produced by luciferase solution with that produced in a control of boiled luciferase solution. I find that a concentrated luciferase solution well shaken with chloroform and filtered, which produced a brilliant light with luciferin, had no oxidative action on phenol, *a*-napthol, para-phenylen diamine, ortol, orcin, hydrochinon, resorcin, pyramidon, phloroglucin, pyrocatechin, gallic acid, benzidin, pyrogallol, gum guaiac, amidol, tannin, or *a*-napthylamine, either with or without H_2O_2. Dubois (*Ann. Soc. Linn. de Lyon*, 1914, lxi, 161) reports oxidation of pyrogallol, tannin, hydroquinon, guaiacol, Tromsdorf reagent, chlorhydrate of diaminophenol, para-phenylene diamine, napthol, napthol *B*, and gum guaiac plus H_2O_2 by a solution of *Pholas* luciferase. Dubois' results are of little value, however, as there is no evidence that the oxidation is due to luciferase rather than the oxidizing enzymes which one finds in cell extracts of all animals, whether luminous or non-luminous.

[2] Harvey, *Am. J. Physiol.*, 1914, xlii, 342; Carnegie Inst. Wash. Pub. No. 251, p. 171, 1917.

by trypsin, as it is difficult to obtain a tryptic digest of protein which does not give the biuret reaction, and the work of Fischer and Abderhalden has shown that certain artificial polypeptids are not digested by pure activated pancreatic juice. We have, then, three possibilities: luciferin is (1) either a natural proteose not attacked by trypsin, or (2) if attacked by trypsin, its decomposition products (presumably amino acids) still contain the group oxidizable with light production, or (3) it is not a protein at all. I believe that luciferin has too many protein characteristics to conform to the last possibility. I have been unable to oxidize with light production various mixtures of amino acids (from beef and casein) by means of luciferase and consequently am led to believe that luciferin is a natural proteose soluble in absolute alcohol and not digestible by trypsin.

Dubois believes *Pholas* luciferin to be a natural albumin with acid properties. *Cypridina* luciferin could not possibly be regarded as an albumin, but it is very likely that the luciferins of different species of luminous animals differ in certain characteristics. Just as in the case of the luciferases, we know that the luciferins are not identical substances, and only future work can determine in what particulars they differ.

SUMMARY.

Under the term photophelein I have previously included two different substances. One of these is a body, found only in *Cypridina hilgendorfii,* which oxidizes spontaneously *without* light production in the air and corresponds to the luciferin found by Dubois in *Pholas dactylus.* It is dialyzable, thermostable, and may be called *Cypridina* luciferin. In the presence of non-dialyzing thermolable *Cypridina* luciferase (corresponding to my old term photogenin) it oxidizes *with* light production. *Cypridina* luciferin differs from *Pholas* luciferin in that it can not be oxidized with light production by $KMnO_4$, H_2O_2, with or without hemoglobin, or similar oxidizing agents. The other substance is found in many non-luminous animals, is also thermostable, and assists in promoting the luciferin-luciferase reaction. For this substance I propose to retain my old term photophelein. *Cypridina* luciferin will give no light with *Pholas* luciferase and *Pholas* luciferin will give no light with *Cypridina* luciferase.

When *Cypridina* luciferin is oxidized, no fundamental splitting of the molecule occurs, because the product, which I propose to call oxyluciferin, can be readily reduced to luciferin again. This reduction is brought about under conditions similar to those necessary for the reduction of dyes such as methylene blue. Indeed, the change luciferin—> oxyluciferin appears to be very similar to the change leuco-methylene blue—> methylene blue. Oxyluciferin can be reduced to luciferin, which will again give light with luciferase by the reductases of muscle tissue,

liver, etc., or by bacteria; by Schardinger's enzyme of milk; by H_2S or the nascent hydrogen from the action of acetic acid on magnesium; and by palladium black and sodium hypophosphite, all well-known reducing methods. The reaction luciferin $\rightleftharpoons$ oxyluciferin is a reversible one and reduction of oxyluciferin no doubt occurs in animals which burn luciferin within the cell, thus tending for conservation of material. Reduction of oxyluciferin will occur even in presence of luciferase if oxygen is absent. Dilute alkali favors oxidation and dilute acid favors the reduction.

So far as I have been able to determine, luciferin and oxyluciferin have identical chemical properties. Neither is digested by the enzymes malt diastase, ptyalin, yeast invertase, pepsin, trypsin, steapsin, amylopsin, rennin, erepsin, urease, or enzymes occurring in the water extracts of dried spleen, kidney, or liver. Of the above enzymes tried, luciferase is destroyed only by pepsin (probably), trypsin, erepsin, and something in spleen and liver extract. Further properties of *Cypridina* luciferin and *Cypridina* luciferase may be noted from the accompanying tables.

Luciferase is unquestionably a protein and all its properties agree with those of the albumins. Although used up in oxidizing large quantities of luciferin, it behaves in many ways like an enzyme and may be so regarded.

Luciferin, on the other hand, is not digested by proteolytic enzymes, is dialyzable, almost but not completely precipitated by saturation with $(NH_4)_2SO_4$, and is soluble in absolute alcohol, acetone, and some other organic solvents, but not in the strictly fat-solvents like ether, chloroform, and benzol. There are, however, certain CO-NH linkages which are not attacked by proteolytic enzymes and some peptones soluble in absolute alcohol, so that these two characteristics do not bar it from the group of proteins. Luciferin, in fact, has many properties in common with the proteoses and peptones and may be provisionally placed in a new group of natural proteins on the borderland between the proteose and peptones.

TABLE 1.

Enzyme solution.	Action of enzymes on luciferase, at 38° in water (unless stated otherwise).							Action of enzymes on luciferin at 38° C. kept in absence of oxygen.				
	Digestive power tested.	Preservative.	Light with luciferin after—				Remarks.	Preservative.	Light with luciferase after—			Remarks.
			24 hours.	48 hours.	72 hours.	96 hours.			18 hours.	24 hours.	48 hours.	
Maltdiastase (Merck)	Starch digestion	None	Brilliant					None	Good			
Same, boiled	No digestion	Do.	Do.					Do.	Do.			
Salivary diastase	Starch digestion	Do.	Do.					Do.			Good	Repeated with same result.
Same, boiled	No digestion	Do.	Do.					Do.			Do.	
Yeast invertase	Cane-sugar digestion	Toluol			Bright		Repeated twice with same result.	Do.		Good		
Same, boiled	Do. Do. Do.	Do.			Do.			Do.		Fair		
Pepsin (Merck U. S. P.) in less than 0.2 per cent HCl.	Coag. albumen digestion	Xylol	Good		Good				Fair			Solution neutralized before testing.
Same, boiled	No digestion	Do.	Brilliant		Brilliant				Do.			0.2% HCl used.
Trypsin (Fairchild Bros. and Foster)	Fibrin but not olive-oil digestion.	Toluol	Faint	Neg								
Same, boiled	No digestion	Do.	Brilliant	Bright								
Pancreatin (Parke, Davis Co.) in 0.5 per cent Na_2CO_3.	Albumen and starch digestion.	Xylol	Fair	Faint		Faint	Luciferase conc. Repeated twice with neutral pancreatin and same result.	Toluol			Bright	Neutral pancreatin used.
Same, boiled	No digestion	Do.	Brilliant	Brilliant		Brilliant		Do.			Bright	
Pancreatin, (Merck)	Fibrin and starch digestion	Toluol	Neg					Do.			Do.	
Same, boiled	No digestion	Do.	Bright					Do.			Do.	
"Steapsin" (Diges. Fer. Co.)	Fat, fibrin, and starch digestion.	Toluol	Faint	Neg			Note that this solution contains trypsin.	Do.			Do.	Repeated twice with same result.
Same, boiled	No digestion	Do.	Brilliant	Bright				Do.			Do.	
Rennin (Chr. Hansen)	Coagulated milk	None	Bright					None	Fair			
Same, boiled	No coagulation	Do.	Do.					Do.	Do.			
Erepsin (Diges. Ferment Co.)	Starch and peptone, but not fibrin or albumen digestion	Toluol	Fair	Neg			Repeated with same result.	Toluol			Do.	Repeated twice with same result.
Same, boiled	No digestion	Do.	Bright	Bright				Do.			Do.	
Arlco urease	Urea hydrolyzed	Do.	Do.					None	Good			
Same, boiled	No action	Do.	Do.					Do.	Do.			
Spleen (calf) subs. (Dig. Fer. Co.).	Olive-oil but not fibrin digestion.	Do.	Good	Faint	Very faint	Neg		Do.		Good		
Same, boiled	No digestion	Do.	Bright	Bright	Bright	Bright		Do.		Do.		
Kidney (beef) subs. (Dig. Ferment Co.)	Fibrin or oil not digested	Do.	Good	Fair	Fair	Fair						
Same, boiled	No digestion	Do.	Do.	Do.	Do.	Do.						
Liver (hog) subst. (Dig. Ferment Co.)	Olive-oil but not fibrin digestion	Do.	Do.	Faint	Faint	Neg						
Same, boiled	No digestion	Do.	Bright	Bright	Bright	Bright						

TABLE 2.—*Properties of photogenic substances.*

Property.	Luciferase.	Luciferin.
Salting out—		
By saturation NaCl	Not precipitated	Not precipitated.
By half-saturation $MgSO_4$	Do	Do.
By saturation $MgSO_4$	Nearly completely precipitated.	Partially precipitated.
By saturation $MgSO_4$+acetic acid		Do.
By half-saturation $(NH_4)_2SO_4$	Slightly precipitated	Not precipitated.
By saturation $(NH_4)_2SO_4$	Completely precipitated	Nearly completely precipitated.
By saturation $(NH_4)_2SO_4$+acetic acid.		Do.
Solubility in—		
Methyl alcohol	Insoluble	Soluble.
Ethyl alcohol	Do	Do.
90 per cent	Do	Do.
70 per cent	Do	Do.
50 per cent	Slightly soluble	Do.
Propyl alcohol	Insoluble	Do.
Isobutyl alcohol	Do	Fairly soluble.
Amyl alcohol	Do	Slightly soluble.
Benzyl alcohol	Do	Soluble.
Acetone	Do	Fairly soluble.
90 per cent	Do	Soluble.
70 per cent	Slightly soluble	Do.
50 per cent	Fairly soluble	Do.
Ethyl acetate	Insoluble	Do.
propionate	Do	Fairly soluble.
butyrate	Do	Do.
valeriate	Do	Slightly soluble.
nitrate	Do	Very slightly soluble.
Glycerine	Do	Soluble.
Glycol	Do	Do.
Ether	Do	Insoluble.
Chloroform	Do	Do.
Carbon disulphide	Do	Do.
tetrachloride	Do	Do.
Benzol	Do	Do.
Toluol	Do	Do.
Xylol	Do	Do.
Petroleum ether	Do	Do.
Anilin	Do	Do.
Glacial acetic acid	Do	Fairly soluble.

TABLE 3.—*Properties of photogenic substances.*

Property.	Luciferase.	Luciferin.
Alkaloidal reagents:		
Phosphotungstic acid	Completely precipitated	Very nearly completely precipitated.
Phosphotungstic and acetic acids		Do.
Phosphotungstic acid and HCl		Completely precipitated.
Tannic acid	Nearly completely precipitated.	Nearly completely precipitated.
Tannic and acetic acids		Do.
Tannic acid and HCl		Do.
Picric acid	Nearly completely precipitated.	Not precipitated.
Picric and acetic acids		Do.
Picric acid and HCl		Do.
$K_4Fe(CN)_6$ and acetic acid		Do.
Heavy metal salts:		
Basic lead acetate	Completely precipitated	Not completely precipitated.
Neutral lead acetate	Nearly completely precipitated.	Do.
Neutral lead acetate and acetic acid.		Not precipitated.
Mercuric chloride	Not precipitated	Not completely precipitated.
Mercuric chloride and acetic acid		Almost completely precipitated.
Uranyl nitrate and acetic acid		Not completely precipitated.
Acids and alkalies:		
NaOH	Not precipitated	Not precipitated.
NH_4OH	Do	Do.
Acetic acid	Do	Do.
H_2CO_3	Do	Do.
Trichloracetic acid	Do	Do.

V.

THE OVARY OF FELICHTHYS FELIS, THE GAFF-TOPSAIL CATFISH: ITS STRUCTURE AND FUNCTION.

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Four plates, one text figure.

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Figures 1 and 2 of this paper were drawn by Mr. E. A. Morrison under a grant from the Carnegie Institution of Washington. The photographs were made by the author.

THE OVARY OF FELICHTHYS FELIS, THE GAFF-TOPSAIL CATFISH.

By E. W. Gudger.

INTRODUCTION.

In previous papers (Gudger, 1916, 1918) I have described in some detail the natural history of this interesting fish, and especially the remarkable habit of the male of carrying in his mouth the eggs until they are hatched and the larvæ until they are capable of fending for themselves. In the present paper attention will be called to the female and her distinctive organ, the ovary, which is at all seasons an interesting structure and at the time of egg-extrusion a most remarkable one. Furthermore, the structure of the ovary of the gaff-topsail is all the more worth study since practically no investigation has been made on this organ in any siluroid, as will be seen when the historical portion of this paper is reached. Moreover, there is but one figure known of the ovary of a catfish. This will be reproduced and described later.

A large amount of material and data is at hand for a description of the ovary of the gaff-topsail. All this was incidentally accumulated in the Beaufort (North Carolina) laboratory of the United States Bureau of Fisheries, while collecting material and making notes on the habits and embryology of this fish; and now that work is being done on the ovary, several questions arise to which (unseen before) neither material nor notes give answer.

THE BREEDING FEMALE GAFF-TOPSAIL.

The "ripe" female gaff-topsail catfish, *i. e.*, with eggs ready for extrusion or approaching that condition, may be readily recognized, for her belly becomes greatly distended. Figure 2, plate 2, shows a non-breeding female gaff-topsail and is fairly slender in general outline, but unfortunately I have no contrasting figure of a "ripe" breeding female, showing the balloon-like expansion of the abdomen.

The second structure, enabling one easily to recognize the fish ready to extrude eggs, is the extremely vascularized, swollen, and protuberant oviducal orifice. This is an infallible sign of ripeness. Males *may* show a swollen abdomen, due to feeding on fish and crabs, although even at the breeding season they do not have such exaggerated genital orifices, but in the female this is so much enlarged as almost to obscure both the anal and urinary orifices between which it is placed.

The following measurements will help the reader to realize the relative sizes of adult breeding females: one was 24.5 inches long over all, girth 13 inches; another 23.5 inches long, 13.5 girth; two fish were 23.5 and 24 inches in length, 13 around; one 22 long by 14 around; and greatest of all, 25 inches over all by 19 in circumference. This last fish, huge as she was, was not quite ripe, since the eggs would not come away when pressure was applied to the abdomen. In such a specimen the great distension of the body-walls, due to the swelling ovary, tends to thin these down markedly.

In figure 3, plate 2, we have a poor photograph of the 24.5 individual with a girth of 13 inches, as noted above, dissected to show how much of the body-cavity of the fish is occupied by the ovary. This organ was 7.5 inches long, 9.75 inches at its greatest girth, and, although it occupied almost all of the abdominal cavity, was not yet ripe. It weighed 435 grams. In front is seen the stomach crowded close up to the cardiac region, while posteriorly the much reduced intestine emerges from the space between the two lobes of the ovary in which it lies.

THE OVARY VIEWED EXTERNALLY.

In figure 1, plate 1, an ovary drawn natural size and viewed from the ventral aspect, this organ is seen to consist of two lobes confluent behind and leading into a single oviduct opening between the anus and the urinary orifice. It is of the normal teleostean type, abnormal in size, and in the size of its giant eggs, which may be easily seen through the (at this stage) relatively thin walls. This ovary is 7.5 inches in greatest length and 3.5 inches wide, and has one lobe longer than the other, as is generally the case. It is from the 24.5-inch female with a girth of 13 inches, shown with open abdomen in figure 3, plate 2. When drawn, the accidental cut in the ovisac seen in the photograph was not represented.

The ovary is slung to the dorsal wall of the body-cavity by the mesovarium, or double layer of peritoneal epithelium. On their dorsal surfaces, the 2 lobes of the ovary are, except at their anterior ends, closely applied, forming a comparatively flattened surface as may be seen in E, figure 8, plate 4. In case the anterior horns of the ovary diverge, the peritoneal covering forms a transparent sheet stretched dorsally across the space separating them. On the ventral surface, however, the lobes curve sharply inwardly and dorsally, leaving between them a region inverted V-shaped in transverse section. Dorsally the ovary is closely applied to the kidneys, and ventrally the now much reduced intestine is closely applied to the base of the inverted V, between the two lobes of the ovary, tightly held in the seam where the peritoneal coats of each ovisac are closely applied.

As the breeding season approaches, the eggs and their containing ovisacs grow larger, more and more filling the cavity of the abdomen

and crowding the stomach forward (unfortunately my notes make no mention of the liver, which presumably shrinks in size somewhat), and the intestine becomes greatly reduced, stringy, and filled with mucus. Apparently the female does not feed as the breeding season approaches its height, or if she does feed it must be on very small objects, since there is no room in the stomach for the common, but bulky, diet of crabs.

The ovary is richly supplied with blood-vessels which descend from the hinder region of the kidneys and reach the ovary at about the point where the ovisacs unite to form the short oviduct. Here the vessel forks, one branch going to the oviducal part and the other to the ovisacs.

INTERNAL STRUCTURE OF THE OVARY.

The general internal arrangements of the ovary are, in the main, correctly foretold by the external appearance—two hollow pouches closed in front and confluent behind into a short, common oviduct whose length and diameter are approximately equal. However, each ovisac from its point of junction to its forward extremity is divided roughly into three nearly equal parts, of which only the foremost bears eggs. The posterior third is raised up into closely placed longitudinal folds or plaits, which at the time for oviposition allow for the great distention due to the passage of the 18 to 20 mm. eggs. These eggs are developed in the anterior third of the ovisac, each egg in a follicle swung by a long pedicel.

Thus we see that only the anterior third of each ovisac produces eggs, while the hinder third is an oviduct pure and simple, which unites with its fellow to form the short, unpaired tube leading to the genital aperture. However, between the plicated hinder section of the gonad and the anterior ovigerous region is a debatable land which belongs hardly to either and yet to both. As the plicæ extend forward they decrease in height and, losing their distinctive form as mere folds, become covered on both sides with small eggs. Further forward they become mere ridges, but distinct enough for the eggs to show up in rows. Still further forward the ridges disappear, and this region of small eggs finally disappears into that wherein the functional eggs are formed. These structures may be fairly well seen in figures 4 and 5 of plate 3, in figure 6 of plate 4, and in c of figure 8, plate 4. In the forward region the arrangement of the eggs in longitudinal rows is completely lost—there is no longer any definite arrangement whatever.

This division of the ovisacs into anterior egg-bearing and posterior plicated oviducal parts with an intermediate region is not true of immature and spent ovaries only. As the time for oviposition approaches, the eggs in the forward ends of the sacs become greatly enlarged, while the debatable region develops great numbers of small eggs which come away readily and which seem never to become func-

tional. The regions of large functional eggs and of small functionless eggs are marked off by an irregular but well-defined line. Forward of this line are found scattered eggs, large and small, destined to become mature and oviposited; behind it are closely aggregated large masses of small eggs, of whose function I am entirely ignorant. These regions and their eggs are very clearly seen in figure 1 of plate 1, an external view, and figure 4 of plate 3, an internal view of the same ovary. However, as the functional eggs ripen, the pedicels of those lying near the line of demarcation greatly lengthen and the eggs pushed backward by the growing ones in front become crowded in among the smaller eggs in the intermediate section, as may be clearly seen in figure 4 of plate 3 and figure 6 of plate 4.

Thus it is that in spent or immature ovaries the ovigerous and oviducal portions of the ovary are about equal in length, while in the pregnant organ the developing eggs and the distention brought about thereby cause each ovisac to become apparently divided into two sections of about equal length—the ovigerous section being in front and the oviducal portion behind. These phenomena will be made clearer in the section dealing with the changes of size.

When the ripe eggs break from their follicles they fall into the lumen of the ovisac and thence pass into the rugose oviducal section and so to the exterior. The follicles are left behind and are very prominent in an ovary from which the eggs have recently been extruded. This is shown very clearly in figure 5 of plate 3. Shortly after the breeding season these emptied follicles entirely disappear, are completely resorbed, leaving only the 1 to 5 mm. eggs which will develop into those of next season's laying. Small eggs in small vesicles standing on short pedicels are found under and around the pedicels of the large eggs; they line the whole of the interior of the ovigerous portion of each sac.

The walls of the ovary are fairly thick (about one-eighth inch) and tough in the resting stage, but they are very distensible, and, as the huge eggs develop, the walls become as thin and (especially in the anterior section) as transparent as oiled paper. The walls of the ovary are composed of two layers, the inner or germinal layer, from which the eggs and their follicles are developed, and the outer or peritoneal envelope. In ovaries which have been in weak formalin for 8 or 10 years, the two layers can be easily separated.

To bring in the food materials required for building up the large number of these giant eggs, a generous blood supply must be provided. The ovarian artery descends from the hinder region of the kidneys about the level of the posterior third of the ovary, but before reaching this organ it divides, one branch going forward to the egg-forming section, the other backward to the oviducal portion. There seems to be some sort of mechanism to send the major portion of the blood to that part

of the ovary needing it. While the eggs are in the process of making, the greater volume of blood goes to and through the forward branch, but as the breeding season approaches more and more blood is sent to the hinder portion, the plaits of which become blood-red in color and very much enlarged, while the genital papilla increases from a small and very indistinct pore to a genuine papilla.

The eggs are very large, ranging from 17 to 22 mm. in diameter when ripe. The investing follicles are everywhere permeated with a network of blood-vessels, and just before the eggs ripen the whole anterior section of each sac is almost blood-red in color. Examined more closely each follicle shows beautifully its mesh of interwoven blood-vessels. This is faintly shown in figures 5 and 6, plates 3 and 4. When the follicles burst to set the eggs free into the lumen of the sac a good deal of blood is lost, and if at this time the fluid from the oviduct is examined microscopically large numbers of white and red corpuscles will be found mixed with small eggs, which have been torn off and are moving toward the exterior. It is surprising that there is not more bleeding. Whether the elastic fibers of the follicles by their contraction check the bleeding, or whether there is some secretion in the blood which causes contraction of the muscular fibers of the arteries, can not be said, but the bleeding shortly stops and the clots are a marked feature of the blood-vessels in the evacuated follicles.

Probably the oviducal portion of the ovary secretes a mucus as a lubricant to aid in the outslipping of the eggs, a thing quite necessary when one considers the size of the eggs in proportion to the normal size of the exit channel.

RELATIVE SIZES OF IMMATURE, SPENT, AND RIPE OVARIES.

It is desirable to make comparison of the sizes, relative and absolute, of ovaries in the three stages indicated above. The large amount of material at hand is presented in the form of the tables appended, which, however, form a fairly graded whole, though in only a few cases can data be given for the size of the fish and the date of capture. These specimens have all been in formalin from 7 to 10 years, and hence their measurements are somewhat less than when they were alive and fresh. The weights are accurate, the lengths and circumferences approximate to within a few millimeters.

IMMATURE OVARIES.

In the ovaries noted in table 1, there was no evidence that eggs had ever come to maturity and no empty follicles. In the posterior section of the ovigerous portion of the ovisacs were minute straw-colored eggs, while in the forward part the eggs had the yellow of real yolk. The largest eggs ranged from 2 to 3 mm. in diameter, the average being about 2.5 mm. No difficulty was had, even in these

small ovaries, in telling where one region left off and the other began; the line between them, while not a definite and straight one, was very clearly discernible.

TABLE 1.—*Immature ovaries.*

No.	Weight.	Length.	Girth.	Notes on ovaries and eggs.	
				Posterior section.	Anterior section.
	grams.	*mm.*	*mm.*		
1	2.1	45	35	Eggs below 0.5 mm. diam.....	Eggs up to 3 mm. diam.
2	4	56	36	Eggs below 0.5 mm. diam.....	Eggs up to 2.5 mm. diam.
3	7.4	57	57	Eggs below 0.6 mm. diam.....	Eggs up to 2.6 mm. diam.
4	7.7	67	67	Eggs microscopic.....	Eggs up to 2 mm. diam.
5	7.8	87	67	Eggs below 0.5 mm. diam.....	Eggs up to 2.5 mm. diam.
6	9.3	67	75	Eggs microscopic.....	Eggs up to 2.5 mm. diam.
Ave.	6.4	63	56	Eggs not above 0.5 mm. diam.	Eggs not above 2.6 mm. diam.

In this table are given measurements for 6 ovaries, varying in weight from 2.1 to 9.3 grams, in length from 45 to 87 mm., and in circumference from 35 to 75 mm., but it should be noted that maximum weight, length, and circumference are not all found in the same ovary. The average weight is 6.4 grams, the mean length 63 mm., while the average girth is 56 mm. On the whole, this set of ovaries presents a fairly graded series, specimens of which are seen in A, B, and C, figure 8, plate 4.

SPENT OVARIES

No. 1 in table 2 is a stray ovary found packed among immature organs of this kind. It is known to be spent because it has a number of torn and empty follicles. As to ovaries 4 to 8, some brief notes were made when the fish were dissected. They were preserved July 26, 1907, and comparison of the measurements made then with the above shows that the preservative has caused but little appreciable shrinking. Their live measurements were 2.25, 2.5, 2.75 inches over all, the average being 2.5 inches. Because these brief data can be given, these ovaries are grouped together in the table. Unfortunately no record was made of the sizes of the fish possessing these organs, but this very fact indicates that they were normal in size, probably running 17 to 22 inches long, with the majority about 18 or 19 inches.

It should be definitely noted here that the line is very clearly marked between the anterior region of functional eggs and the posterior one of small straw-colored eggs. The great masses of 3 to 8 mm. eggs found in this region in ripe ovaries have been swept clean away, leaving this portion of the ovisacs sparsely covered with very small eggs in a single layer set closely upon the germinal epithelium. It will also be noted that some ovaries, and they the largest, have eggs in the forward portion of the ovisacs ranging up to 8 mm. in diameter. The sparse references in my notes indicate that these ovaries were

collected in July, and probably after the 15th, and hence these eggs may be considered as belonging to next year's laying. However, the presence of these large eggs, together with other data, leads to the conjecture that there may be a second laying later in the season.

TABLE 2.—*Spent ovaries.*

No.	Weight.	Length.	Girth.	Posterior eggs.	Anterior eggs.
	grams.	*mm.*	*mm.*		
1	6.1	52	65	Very small.	Up to 3 mm.
2	7.9	55	81	Do.	3.5 "
3	9.7	73	83	Do.	4 "
4	9.8	60	72	Do.	2.5 "
5	12.	58	87	Do.	4 "
6	12.5	62	70	Do.	3 "
7	13.8	67	77	Do.	3 "
8	22.4	70	95	Do.	8 "
9	10	56	74	Do.	5 "
10	11.2	60	70	Do.	3 "
11	13	57	85	Do.	5 "
12	14.2	63	80	Do.	3 "
13	14.8	61	95	Do.	9 "—one 18 mm.
14	18	65	80	Do.	7 "
15	18	75	93	Do.	8 "
16	23.4	72	98	Do.	7 "
17	25.4	83	105	Do.	6 "
18	26.9	67	120	About 1 mm.	
Average..	14.9	64.2	85	Average maximum size of anterior or functional eggs about 5 mm.	

A brief analysis of table 2 shows that ovary No. 1 is about equal in size (weight, length, and circumference) to the average of the immature ovaries. Beginning with a weight of 6.1 grams, there is a progressive increase to No. 18, with a weight of 26.9 grams, the average being 14.9 grams. In length, the extremes are 52 to 83 mm., and in circumference from 65 to 120 mm. Here again we do not find all three maxima in the same organ. Contrasted with these extremes the averages are: for weight, 14.9 grams; for length, 64.2 mm.; for circumference, 85 mm. Unfortunately these ovaries were all dissected before any thought was had of the question of volume, but measurements were made of the largest eggs of next year's crop, the extremes being about 2.5 to 9 mm., with an average of about 5 mm. These ovaries are known to be all spent, by reason of the numbers of torn and evacuated follicles in their forward regions. These were too small to be accurately counted in most of these organs. For a figure of such an ovary, see No. 5, plate 3.

Table 3 lists a number of ovaries collected at various times, but all "spent." All were measured and some dissected. They seemed to be from larger fish than the preceding. It will be seen that the ovisacs are frequently of unequal length, but no data were collected to show whether the right or left was uniformly the longer. No. 5 is probably the ovary shown in figure 5, plate 3. The length of the

ovisac is 93 mm. The opened right one contains 23 empty follicles. Toward the hinder portion of this pouch are seen great numbers of much produced folds of the germinal epithelium of the oviducal section of the egg-bag. Some of these folds were as much as 9 mm.

TABLE 3.—*Spent ovaries—second lot.*

No.	Weight.	Volume.	Length.	Girth.	Miscellaneous notes.
	grams.	*c.c.*	*mm.*	*mm.*	
1	31.1	30	83	105	Left ovisac 10 mm. longer than right one.
2	42.3	43	80	118	Girth of neck of oviduct 70 mm.
3	42.9	.....	85	111	Right ovisac had 13 empty follicles.
4	53.3	.....	82	120	Right ovisac with 31 empty follicles, left with 16.
5	59	.....	93	.....	Right ovisac with 23 empty follicles. See fig. 5, pl. 3.
6	72.8	60	100	132	Left ovisac 95 mm. long. Girth neck oviduct 53 mm.
7	99.1	80	110	150	Largest eggs up to 7 mm. diameter.
8	101.6	.....	103	.....	Right ovisac 90 mm. long and 95 mm. in girth.
Ave.	62.8	53.3	92	122.7	

high. These, however, bear no eggs. Anterior to these and in the middle section of the ovary are the many ruptured follicles of the small straw-colored, non-functional eggs which always break away and pass out with the functional eggs. These latter come always from the anterior region of each pouch. Here are seen their empty follicles and between them eggs 3 to 5 mm. in diameter. These are the beginnings of next year's crop. The wall of this ovary is very much contracted, thick, stiff, leathery, in marked contrast to the thin parchment-like wall of this section distended in the pregnant organ. Equally marked is the delimitation of the ovigerous part of the ovisac into regions bearing functional and non-functional eggs. In the later the larger eggs have the longer pedicels.

The data for No. 4 show the shrinkage possible in a spent ovary. This ovary (which is less than twice as large as the smallest one recorded in this table and but slightly more than half the size of the largest) has 31 empty follicles in the right sac and 16 in the left; it carried 47 eggs, ranging from 17 to 22 mm. in diameter, and yet in the spent state it weighs but 53.3 grams (less than 2 ounces) and is only 82 mm. (slightly over 3 inches) long and 120 mm. in circumference.

Ovary No. 7 of table 4 has had its ovisacs split apart and the left one dissected. This sac has a length of 103 mm. It contains 15 empty follicles, partly resorbed, and about 45 eggs from 7 to 12 mm. in diameter. In the other sac 12 mm. eggs were also found. The finding of empty follicles and such large eggs in the same ovary tends to confirm the hypothesis that there may possibly be two layings in a season.

The largest ovary of the first lot, No. 18, weighed 26.9 grams and measured 67 by 120 mm.; the smallest of the second lot weighed 31.1

grams and measured 83 by 105 mm.; so here again we find no gap in the series. In the present table the extremes in weight are from 31.1 to 101.6 grams, with an average of 62.8; in volume from 30 to 80 c.c., with the average (for 4) 53.3 c.c.; the lengths from 80 to 103, average 92 mm.; while the figures for the circumference run from 105 to 150, averaging (6) 122.7 mm.

HALF RIPE OR NEARLY RIPE OVARIES.

There is now to be considered a small group of half-ripe ovaries, judged to be in this condition because they contain eggs approaching ripeness and because no empty follicles are found in them. They are 7 in number, and all but 2 were dissected as to one or both ovisacs when taken from the fish; hence not all dimensions and volumes can be given. The average weight is about the same as that of the larger-sized spent ovaries, but the average volume, length, and girth of present lot are much greater, on account of the difference in the contents of the two sets of organs. The volumetric measurements are given for two ovaries only. In the case of No. 3, the large measurements are explained by the presence of the 40 eggs of 15 mm. diameter, and for No. 7 by 25 eggs of about 13 mm. The average size of the next year's eggs is about 4.4 mm., and for the unripe eggs of the present year about 14.7 mm.

TABLE 4.—*Half-ripe, or nearly ripe ovaries.*

No.	Weight.	Volume.	Length.	Girth.	Remarks.
	grams.	*c.c.*	*mm.*	*mm.*	
1	36.9	.....	80	.....	Small eggs to 2.5 mm., adult eggs to 14 mm.
2	39.1	.....	78	.....	Small eggs to 4 mm., adult eggs to 10 mm.
3	53	155	103	167	Small eggs to 7 mm., 40 adult eggs to 15 mm.
4	55	.....	.....	.....	Small eggs to 5 mm., adult eggs to 8 mm.
5	61.9	.....	83	.....	Small eggs to 4 mm., adult eggs to 10 mm.
6	81	.....	80	.....	Small eggs to 3 mm., adult eggs to 17 mm.
7	109.2	90	107	170	Ovary crowded with 25 eggs 11 to 13 mm. diam.
Ave.	62.3	122.3	88.5	168.5	Small eggs about 4.4 mm., adult eggs 11 to 13 mm. diam.

It is interesting to note that in these ovaries there are found in the strictly ovigerous section two kinds of eggs: the large dark-yellow eggs nearing ripeness (from 8 to 17 mm. in diameter) and standing on long pedicels, while beneath, on short pedicels sitting close to the germinal epithelium, are the smaller straw-colored eggs of next year's crop. These average from 2.5 to 4 mm. in diameter. In that portion of each sac approaching the plicated folds of the oviduct the eggs are all small, rarely reaching a diameter greater than 2 mm., even in the most advanced organs. For such an organ, see D, figure 8, plate 4.

RIPE OVARIES.

Table 5 deals with ovaries containing ripe eggs, within a few days of extrusion, both eggs and ovaries attaining a size out of all expec-

tation for a teleostean fish, which in hundreds of specimens rarely exceeded 2 feet in total length between perpendiculars. These ovaries were collected during the last 10 days in May or the first week in June, these dates being the limits of the breeding season of this fish at Beaufort (North Carolina), where it was studied. Exact data for time and

TABLE 5.—*Ripe ovaries.*

No.	Weight.	Volume.	Length.	Girth.	Miscellaneous notes.
	grams.	*c.c.*	*mm.*	*mm.*	
1	217.4	197.7	150	184	Right ovisac 16 mm. longer than left.
2	237.6	212	115	235	Right ovisac slightly longer than left.
3	310	265.5	158	230	Left ovisac slightly longer and larger than right.
4	339	307.8	162	213	Fresh; weight 354 g., length 172 mm., girth, 225 mm.
5	395	388.6	155	256	Width 106 mm.
6	401.8	366.2	177	220	Fresh; weight 435 g., length 190 mm., girth 247 mm.
7	433	379.9	170	230	Left ovisac slightly longer than right.
8	469	438	180	243	Girth oviducts at junction, 180 mm.
Ave...	350.4	319.5	158.4	226.4	

for size of fish can be given for 4 specimens only, the labels on the others having gone to pieces. However, the time limits are correct and it should be noted that the largest fish I ever dissected was 25 inches long. As in the preceding cases, the order of arrangement in this table is ascending.

To one accustomed to the ordinary-sized ovaries found in a teleost not exceeding 2 feet in length, the figures in table 5 are almost unbelievable. None of these ovaries, it should be noted, was absolutely ripe, for from none would eggs come away, yet the smallest weighed 217.4 grams and displaced 197.7 c.c. of water. At the other end of the list, No. 8 weighed 469 grams and had a displacement of 438 c.c. The average weight of the 8 is 350.4 grams, the average volume is 319.5 c.c., the mean length 158.4 mm. and the mean circumference 226.4 mm.

Specimen No. 6 was collected May 30, 1909, from a fish 24.5 inches long. When fresh it weighed 435 grams, and after being in formalin for nearly nine years its weight was 401.8 grams. Its length when fresh was 190 mm. (the left lobe being slightly the longer); to-day it is 177 mm. When just excised its circumference at the junction of the oviducts was 175 mm. and its greatest girth 247 mm. Its greatest circumference is now 220 mm. This ovary is seen in ventral view in figure 1, plate 1, and in horizontal longitudinal section in figure 4, plate 3. On being dissected, in order to make the photograph from this latter figure, the right lobe was found to contain 26 eggs, the left 20.

Ovary No. 2 of the above table was dissected and found to have 21 eggs in the right sac and 19 in the left. These eggs in their pediceled

vesicles were all so closely crowded in the anterior section that the sides were flattened. The anterior third of this ovary was greatly distended, the wall of the ovisac being so thin as readily to permit the passage of light. The thin parchment-like peritoneal layer would easily come away from the germinal epithelium. The elongated pedicels of the posterior eggs allowed them to be carried back into the region of non-functional eggs in the intermediate section among which they were found nested.

Ovary No. 3 was also dissected, and its left lobe was found to contain 2 empty follicles and 27 large eggs, about 20 mm. in diameter. Presuming that the right sac contains an equal number, the total for this organ will be 54. The walls of the intermediate section of this ovary are very thick and leathery, measuring 5 mm. from the outside to the lumen of the oviduct. Some of the plicated oviducal folds have considerable height, measuring as much as 7 mm.

We have here studied 5 sets of ovaries, making a fairly complete series, from very small to very large. We had first very small, immature ovaries; next small, spent ovaries; next larger, spent ovaries; then half-ripe ovaries, and lastly these organs with eggs nearly ripe enough for extrusion. In Nos. 1, 2, 3 the ovaries are comparatively thick-walled, while in Nos. 4 and 5 the great enlargement brought about by the growing eggs is shown in the distention and thinning down of the walls of the anterior section.

SIZE OF EGGS AND NUMBER FOUND IN OVARY.

In the paper elsewhere referred to (Gudger, 1918) the question of the size of the ripe eggs has been considered in considerable detail. Also, in the section dealing with ripe ovaries, considerable data have been given touching the matter of the size of the eggs. Hence it will be sufficient here to give the mere facts.

From the large number of measurements made, the following may be selected as thoroughly representative. Of live eggs, 138 measured as follows: longest diameter, 17.5 mm. minimum to 21.5 mm. maximum. The largest numbers were 39 eggs of 19 mm. diameter; 38 of 20 mm.; 19 of 21 mm.; 22 of 21.5 mm.; average for 138 live eggs, 18.8 mm. Of preserved eggs, 189 measurements were made; the smallest were 16.5 mm. and the largest 22 mm. in diameter. The largest numbers here were 34 eggs of 19 mm., 64 of 20 mm., and 49 of 21 mm. The average for 189 eggs was 20 mm., and the general average for 327 eggs was 19.5 mm. in diameter. The largest numbers of eggs were found in the 19, 20, and 21 mm. sets, the 20 mm. eggs being the most numerous—102 out of 327 eggs measured. Normal eggs in early stages of development may be seen in figure 7, plate 4.

In the section dealing with ripe ovaries, the numbers of eggs found therein and nearly ready for extrusion have been noted. Specimen

No. 6 of the table, represented in external view in figure 3 of plate 2 and in longitudinal horizontal section in figure 4 of plate 3, contained 46 eggs. Specimen No. 8 of the table, the largest ovary examined, contained only 12 grown eggs in the right sac and 13 in the left. This ovary, for which, unfortunately, no date of capture can be given, since the label has gone to pieces, was, notwithstanding its great size, plainly not ripe. In the right sac were about 20 and in the left about 25 eggs of 10 to 12 mm. in diameter. This tends to confirm the idea previously expressed that there may be more than one egg-laying in a season. If all these eggs were laid in one season, this ovary would have a productive capacity of about 70 eggs.

Ovary No. 2 of the table contained 46 fully grown eggs, while No. 3 gave up 55. However, it is probable that these figures do not represent the maximum. Only one live specimen was studied. A female taken May 27, 1909, was kept in a tank for 4 days. She was then spawned and forced to give up 68 of these huge eggs. This is the largest number of grown eggs I have ever obtained from one fish and is probably the maximum number found in the ovary of any female gaff-topsail catfish.

HISTORICAL ACCOUNTS.

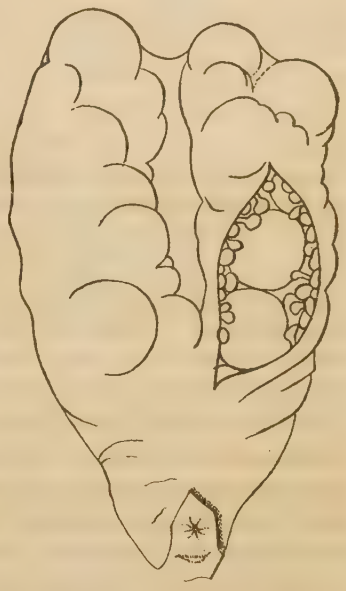
It is surprising to find how little is known about the structure of the ovary of any catfish. So far as the writer knows, no real investigation of this interesting organ has ever been made, and the data now to be presented have been noted by various writers as merely incidental in the course of other investigations on catfishes. As to the ovary of the gaff-topsail, the only two known references will now be briefly considered, and after that the strictly chronological order will be adhered to. In 1884 S. C. Clarke wrote: "The eggs of this species are golden yellow, and of the size of grapes, which they much resemble, in bunches of ten or twelve." And again in 1892, "He slashed it [the gaff-topsail] open with his knife, bringing out a bunch of eggs in form and color like golden grapes." In this connection the attention of the reader is called to figure 6, plate 4.

The oldest direct reference to the ovary of the catfish, which has been found so far, is by Bonnaterre in 1788. He speaks of the eggs of *Silurus ascite* as being "disposed on each side of the abdomen in two packages [ovisacs?], which extend from the diaphragm clear back to the anus." If his brief description is referred to figure 3 of plate 2, of this paper, it will be seen how accurately he wrote 130 years ago.

Next to Bonnaterre, our oldest and most numerous references (for there are no less than 14) are from the Austrian ichthyologist, Rudolph Kner. Writing of Siluridæ from Brazil, in 1858, he gives the interesting data cited below, together with the only known figure of the ovary of a catfish. Of *Arius luniscutis* Cuvier and Valenciennes, he says:

"Figure 6 [reproduced herein as text-figure 1] shows the ovary of the female in natural size [75 mm. long and 44 mm. wide] with the left side partly cut open lengthwise in order to show the already developed large and also the unripe [small] eggs hanging to its walls. This ovary contains 12 to 14 ripe eggs, the largest of 5 lines in diameter, and these like the others, with a small pedicel like an umbilical cord, sit fast on the wall of the ovary and the [thin] yolk skin or sac allows no trace of any embryo to be seen through it. This remarkable difference in the size of the egg at one and the same time and in the same ovary causes one to conjecture that viviparity is not practiced by this fish."

Of *Bagrus mesops* and *Arius quadriscutis*, Kner says that their ovarian structures are identical with those of *Arius luniscutis;* while of *Galeichthys gronovii* he notes that the ovaries are like those in *Arius*, and adds that they are made of 2 thick-walled sacs which are confluent behind into a wide oviduct opening out between the anal and urethral orifices. *Cetopsis goboides* has the long, closed ovarian sacs united along their whole dorsal surfaces by folds of skin to the middle line of the body. At the approach of the breeding season the large eggs cause the ovary to reach forward fully half the length of the body-cavity, but on the other hand at its breeding season the ovaries of *Pimelodus sebæ* occupy the whole length of the body-cavity. *P. bufonius* also has large egg-sacs and *P. laticaudus* at breeding time has a markedly turgid urinogenital papilla.



TEXT-FIGURE 1.—Ovary of *Arius luniscutis*, after Kner, 1858. With the exception of those given herein for *Felichthys felis*, this is the only known figure of the ovary of a catfish.

Kner examined the ovaries of two species of *Auchenipterus*. Of these, *A. ceratophysus* probably was not taken at breeding season, since the ovary occupied about half the body-cavity, but of *A. nodosus* he says:

"The ovaries of the females occupy at the laying season the belly cavity clear to the base of the pectoral fins, are nearly cylindrical, are bound together at their fore ends by a membrane and open out behind into a very wide oviduct."

Finally Kner says of *Centromochlus aulopygius*, *Ageniosus militaris*, *A. brevifilis*, and *Hypothalmus fimbriatus* that they show no remarkable ovarian development, this being probably due to the fact that they were not taken at the breeding season.

The next reference is to Peter Bleeker (1858) and is merely incidental. In describing *Arius arius*, of the Indian Archipelago, he says that

the females retain their eggs for a long time and that as these eggs gradually attain the size of pigeon eggs "the abdomen often swells up above the ordinary." Hardly more definite is Wyman (1859), in writing of siluroids of Surinam:

"The eggs become quite large before they leave the ovaries, and are arranged in three zones corresponding to three successive broods, and probably to be discharged in three successive years: the mature eggs of a *jarra-bakka*, 18 inches long, measure three-fourths of an inch in diameter; those of the second, one-fourth; and those of the third are very minute, about one-sixteenth of an inch."

Not so indefinite, however, is William Turner, the distinguished anatomist of Edinburgh, to whom Rev. Bancroft Boake had sent some specimens of *Arius boakei*, an oral gestator of Ceylon. Among these was one female, of which Turner writes (1867):

"From the appearance of the abdomen it was evident that the ovaries were distended; and on opening into the cavity I found a sac-like ovary on each side of the middle line. Each ovary measured 2½ inches in length, and extended forward almost as far as the pectoral fin, where it formed a rounded free end, whilst posteriorly it was somewhat constricted, and opened by an orifice common to it and its fellow immediately behind the anus. The ovisac contained a very large number of eggs in various stages of growth. Some were like minute granules, others (and these very numerous) like medium-sized shot, whilst a third set equaled in size grapes, or small cherries, and very materially exceeded therefore the size usually attained by the eggs of osseous fishes. These last, only six in number in each ovary, had evidently almost reached the full period of intra-ovarian growth. Each ovum was attached to the inner wall of the ovisac by an independent pedicel, the atrophy of which would necessarily precede the discharge of the egg."

Equally definite is Francis Day (1873), who gives us an account of the structure of the ovary of certain Indian siluroids. In describing the habits of a number of well-known marine catfishes practicing oral incubation, he says:

"Next, the females came under observation. On tracing up the ovisacs it appeared that a very large number of eggs existed in them, but *not all of the same size* [italics Day's]. On the part farthest removed from the outlet the eggs were of full size (about half an inch in diameter), and about 50 in number; whilst other batches of much smaller size existed, evidently to take the place in due time of the larger ones when they had been deposited. The full-sized eggs were each attached to the inside of the ovisac by a pedicel of varying length, distinctly supplied with blood-vessels of considerable size."

The next notice of the ovaries of an Indian catfish is an incidental one from Edgar Thurston in 1900. On the Malabar coast of southwest India he dissected an *Arius* (species undetermined) and found that what he calls the "double uterine cavities" contained respectively 56 and 75 eggs of about 13 mm. in diameter.

At the 1907 meeting of the French Association for the Advancement of Science, Jacques Pellegrin read a paper on buccal incubation

in two catfishes of the genus *Arius* from the fresh waters of Guiana. His paper, published in 1908, contains a good description of the ovary of *Arius fissus*. His statement is as follows:

"Autopsy showed a female (208 mm. in extreme length) with 2 voluminous ovisacs moderately elongated and about equal. The left ovisac was about 50 mm. long, 18 high, and 8 thick. After having been opened it was found to contain eggs in three clearly marked-off stages of development. Principally, in the posterior region there is found a mass of little rounded ovules, more or less ovoidal, grayish-yellow, extremely numerous, and about 0.25 mm. in diameter. On the 'face externe et inferiere' and between the large ovules are found some hundreds of medium-sized ovules, more or less ovoidal, grayish-yellow, having a large diameter of 1.5 mm. and a small diameter of from 1 to 1.5 mm. All the rest of the gland is filled with enormous ovules, rounded at maturity, dark green, tightly compressed against one another and superimposed in three ranks like the seeds of a pomegranate, to the number of a score, and having a diameter of about 6 to 7 mm. The right ovisac being exactly similar, there were on the walls some 40 eggs which were deemed ready for laying."

Last of all, Willey, writing of Boake's *Arius falcarius* (*boakei*) of Ceylon, says (1910):

"The ovaries of an adult female contain a very great number of eggs in different stages of growth, but of these only a few become mature at a time and there is a great contrast in size between the mature and the immature ovarian ova. In one case there were only 10 large eggs in the right ovary and 8 in the left. In another there were 21 large eggs in the right and 24 in the left."

For another teleost, but one far removed in time and place from *Felichthys*, Weber (1908) may be quoted concerning *Apogon beauforti*, a Cheilodypterid fish from New Guinea:

" . . . . the forward part of whose ovary bore large eggs in long stalked follicles, while the hinder part encompassed numberless small eggs, between which only here and there a large egg lay. Perhaps it is the rule that only a small number of eggs become ripe while the others undergo resorption."

Bearing on some of the points referred to in the preceding notes, Louis Agassiz (1868) may be quoted on the turtles of the Amazon:

"[They] always contain several sets of eggs. Those which will be laid this year are the largest; those of the following year are next in size; those of two years hence still smaller; until we come to eggs so small that it is impossible to perceive any difference between their various phases of development."

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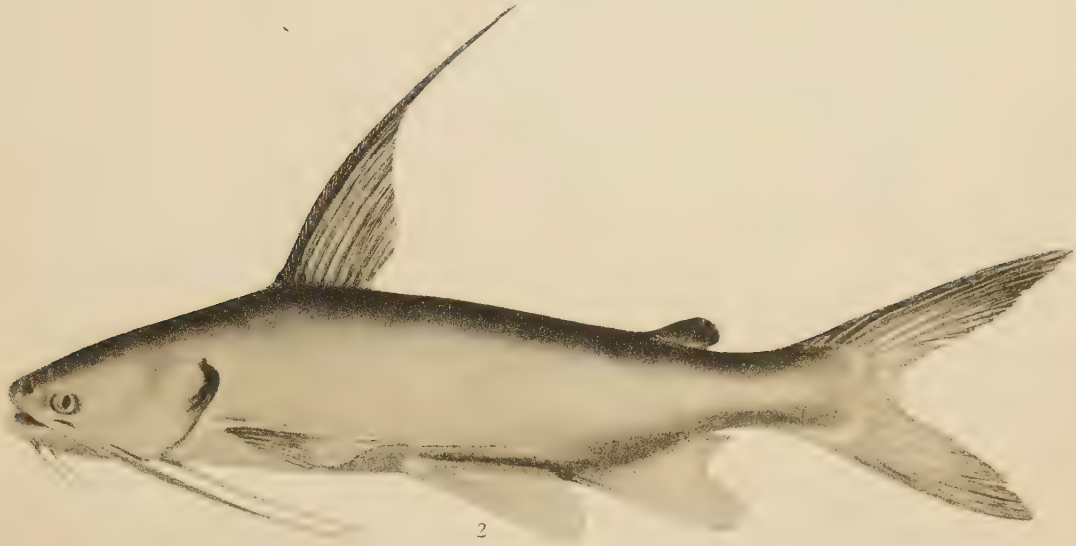
1. Ventral view of ovary of Gaff-topsail catfish, 24 inches long, drawn from life, natural size.



2

3

2. Lateral view of female Gaff-topsail, 19.25 inches long, drawn from life.

3. Female Gaff-topsail dissected to show ovary *in situ*. For figure of ovary natural size, see plate 1.

4

5

1. Internal view of ovary shown in plate 1, and in figure 3, plate 2. Horizontal longitudinal section. Preserved specimen.

5. Opened live ovisac magnified to show torn follicles in middle and anterior regions, next year's eggs in forward section, and plicated folds of oviduct.

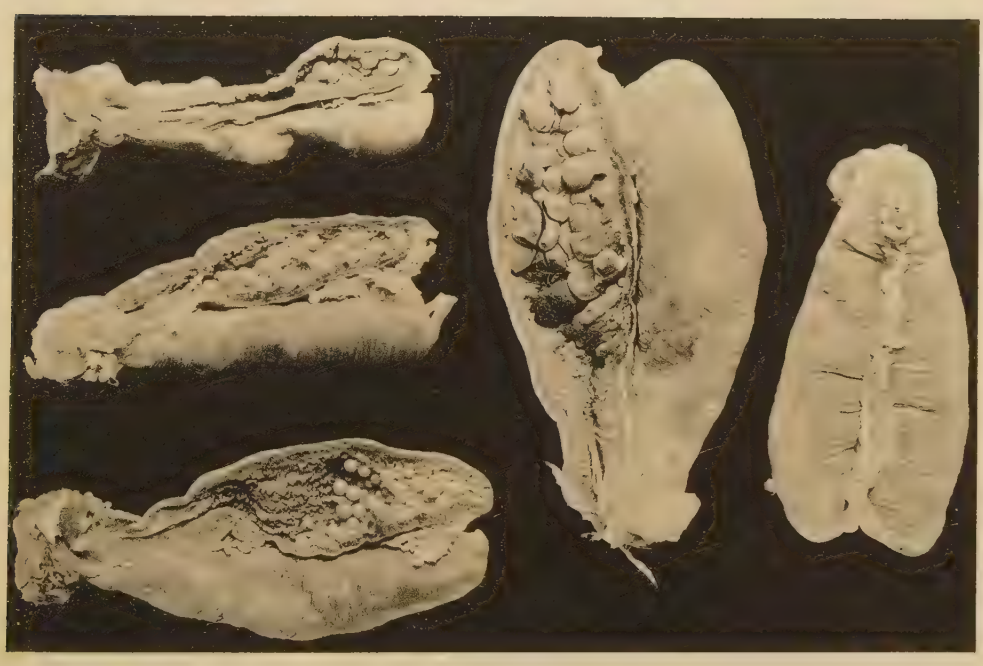
6

7

A

B

C



8

D E

6. Ovary with one side opened to show ripe eggs, small eggs of intermediate zone, and plications of oviducal section. Reduced about one-half.

7. Eggs with embryos normal size.

8. A, B, C. Immature ovaries in ascending order of age and development.
D. An ovary about half ripe.
E. An ovary in about the same stage seen in dorsal view.
Preserved material.